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EARLY-LIFE SELENIUM STATUS AND COGNITIVE DEVELOPMENT

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Cover picture ‘Selenium on my mind’ by Helena Skröder Löveborn.

In 1817, the chemist Jöns Jacob Berzelius noticed a mineral which he first thought was tellurium. He realized that the substance was a new element, and decided to name it **selenium** after the Greek word Σελήνη, selènè (moon), in a similar manner to tellurium, named after the latin word for earth, tellus.

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EARLY-LIFE SELENIUM STATUS AND COGNITIVE DEVELOPMENT

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Many of the things we need can wait.
The child cannot.
Right now is the time his bones are being formed,
his blood is being made
and his senses are being developed.
To him we cannot answer "Tomorrow".
His name is "Today".

- Gabriela Mistral, 1948

I dedicate this work to past, present, and future children
suffering the silent epidemics of micronutrient malnutrition

ABSTRACT

Selenium is an essential element that is found in food sources such as meat, fish, and cereals. The essentiality of selenium was demonstrated in the 1950s, and the interest in its health effects has been growing ever since. Deficiency is common world-wide, particularly in Europe and south-eastern Asia. It has been estimated that 0.5-1 billion people could be selenium deficient.

Previous studies regarding health effects of selenium have focused on the impact of deficiency for the risk of cancer, cardiovascular disease, and decreased fertility and immune function. Lately, the importance for brain function has also become the focus of many studies assessing potential protection against cognitive decline and certain neurodegenerative diseases, such as Alzheimer's and Parkinson's disease. However, little is known about the importance for early-life development. In particular, the role of selenium in cognitive development has not been studied, even though the brain is one of the organs with highest selenium priority at deficient intake levels. Therefore, the overall aim of this thesis was to assess whether selenium status in early life is important for cognitive development.

The studies included in this thesis were based on data from a large mother-child cohort in rural Bangladesh. The cohort was nested in a randomized food and micronutrient supplementation trial that was established in 2001-2003, called the Maternal and Infant Nutrition Intervention, Matlab (MINIMat). Women were recruited to this nested cohort early in pregnancy (at pregnancy testing), and donated urine and blood samples continuously throughout pregnancy. The subsequently born children were divided into two groups for different outcome assessments. For cognitive assessment, children were followed-up at 1.5, 5, and 10 years, while assessment of immune function and various effect biomarkers was performed in the other group of children at 4.5 and 9 years of age.

To evaluate the role of selenium in cognitive development, concentrations of the element were measured in urine and blood from the pregnant women, and also in blood, urine, and hair from the Bangladeshi children at different ages (n=223-1408). Results from the group of children who donated blood, urine, and hair, demonstrated that also hair selenium could be used for assessment of selenium status in the present population. Using multivariable-adjusted regression analyses, we found that adequate selenium status during pregnancy seemed important for the children's cognitive development. Children born to mothers with higher selenium status performed better on the cognitive tests at 1.5, 5 and 10 years of age. Also the selenium status during early childhood seemed to be important for the cognitive abilities at the 5- and 10-year follow-ups. There was an indication of an upper limit for the positive association, in line with the narrow therapeutic interval for selenium.

Assessment of influential factors for the selenium biomarkers indicated that exposure to arsenic and cadmium (both strong pro-oxidants) changed the distribution of selenium between different biological compartments (or *vice versa*). Importantly, malnourished

children seemed to retain more selenium, supporting that the regulation of selenium occurs through changes in urinary excretion also in children.

To conclude, this research, based on different biomarkers of selenium status and comprehensive testing of cognitive abilities in large samples of children at 1.5 (n=729), 5 (n=1260) and 10 (n=1408) years of age provides substantial, new evidence of the importance of adequate early-life selenium status for brain development. Similar studies in other populations, as well as research on efficient ways to improve inadequate selenium status without risking selenium toxicity, are warranted.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals I-IV:

I. **Skröder H**, Hamadani J, Tofail F, Persson LÅ, Vahter M, Kippler M. Selenium status in pregnancy influences children's cognitive function at 1.5 years of age. *Clinical Nutrition*. 2015 Oct;34(5):923-30.

II. **Skröder H**, Kippler M, Nermell B, Tofail F, Levi M, Rahman SM, Raqib R, Vahter M. Major limitations in using element concentrations in hair as biomarkers of exposure to toxic and essential trace elements in children. *Environmental Health Perspectives.* 2017 Jun;29:125(6):067021.

III. **Skröder H**, Kippler M, Tofail F, Vahter M. Early-life selenium status and cognitive function in Bangladeshi children. *Environmental Health Perspectives.* 2017 Nov;125(11):117003.

IV. **Skröder H**, Kippler M, De Loma J, Raqib R, Vahter M. Predictors of selenium biomarker kinetics in 4-9-year-old Bangladeshi children. *Environment International*. 2018 Dec:121(1):842-51.

LIST OF SCIENTIFIC PAPERS NOT INCLUDED IN THIS THESIS

- De Loma J, **Skröder H**, Raqib R, Vahter M, Broberg K. Arsenite methyltransferase (AS3MT) polymorphisms and arsenic methylation in children in rural Bangladesh. *Toxicology and Applied Pharmacology.* 2018 Oct;357:80-87.

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- **Skröder H**, Hawkesworth S, Kippler M, El Arifeen S, Wagatsuma Y, Moore SE, Vahter M. Kidney function and blood pressure in preschool-aged children exposed to cadmium and arsenic - potential alleviation by selenium. *Environmental Research*. 2015 Jul;140:205-13.

* Authors contributed equally

CONTENTS

LIST OF ABBREVIATIONS

AI	Adequate intake
As	Arsenic
ADHD	Attention Deficit Hyperactivity Disorder
AS3MT	Arsenite methyltransferase
BSID-II	Bayley Scales of Infant Development, 2nd edition
$-CH_3$	Methyl group
CI	Confidence interval
DMA	Dimethylarsinic acid
EAR	Estimated average requirement
$[(GS)_2AsSe]^-$	Seleno-bis (S-glutathionyl) arsinium ion
GPx	Glutathione peroxidase
GS-Se-GS	Selenodiglutathione
GW	Gestational week
H_2Se	Hydrogenselenide
iAs	Inorganic arsenic
icddr,b	International Centre for Diarrhoeal Disease Research, Bangladesh
ICP-MS	Inductively coupled plasma mass spectrometry
INMT	Indolethylamine N-methyltransferase
IQ	Intelligence quotient
LOD	Limit of detection
MMA	Methylarsonic acid
RDA	Recommended dietary allowance
ROS	Reactive oxygen species
SAM	S-Adenosyl methionine
Se	Selenium
SECIS	Selenocysteine insertion sequence
SeCys	Selenocysteine
Se-homoCys	Selenohomocysteine
SeMet	Selenomethionine
SeO_3^{2-}	Selenite

SeO_4^{2-}	Selenate
T3	Triiodothyronine
T4	Thyroxine
TMSe	Trimethylselenonium ion
TRH	Thyrotropin-releasing hormone
TrxR	Thioredoxin reductase
TSH	Thyroid-stimulating hormone
UL	Tolerable upper intake level
WPPSI	Wechsler Pre-school and Primary Scale of Intelligence
WISC-IV	Wechsler Intelligence Scale for Children, 4^{th} edition

1 INTRODUCTION

Micronutrient malnutrition is often referred to as the “hidden hunger” because the consequences are not always visible. In the past, this has mainly concerned four micronutrients: vitamin A, zinc, iron, and iodine. However, other micronutrients with prevalent deficiency world-wide, such as selenium, are also of great importance. It has been estimated that 0.5-1 billion people world-wide are selenium deficient (Combs 2001). The main areas affected are Europe and large parts of south-eastern Asia (Fairweather-Tait et al. 2011). However, there are also seleniferous areas in e.g. western U.S., Canada and parts of China and Russia, where the selenium intake can reach even toxic levels. The interval between the essentiality and toxicity of selenium is rather narrow.

The focus of this thesis is on the potential importance of early-life selenium status for cognitive development. Although many have studied the association between selenium status and cognitive decline in elderly, less is known about the impact of selenium for cognitive function earlier in life. This is particularly important since adequate nutrition during pregnancy and childhood is crucial for normal brain development, laying the foundation for the development of cognitive skills, motor function, and socio-emotional skills throughout both childhood and adult life.

2 BACKGROUND

2.1 SELENIUM IN HUMANS

Selenium is a non-metallic micronutrient that was first discovered by the Swedish chemist Jöns Jacob Berzelius in 1817. The essentiality of selenium was demonstrated in the 1950s (Schwarz and Foltz 1957), and the interest for its positive health effects has been growing ever since.

2.1.1 Selenoproteins

Selenium exerts its biological effects through different selenoproteins. In total, 25 selenoproteins have been identified in the human proteome (Burk and Hill 2015; Kryukov et al. 2003), but not all of them have been functionally characterized. In the selenoproteins, selenium is incorporated in the form of selenocysteine (Figure 1), in which the sulfur atom of cysteine is replaced by a selenium atom. This results in a lower pK_a and higher reactivity of the functional selenol group, compared to a thiol group. All selenoproteins contain one selenocysteine residue, except for selenoprotein P, which contains ten such residues. This selenoprotein constitutes the major circulating and storage form of selenium, and seems to play a role in antioxidant protection (Burk and Hill 2015).

Several of the other selenoproteins also exhibit antioxidative properties, such as glutathione peroxidases (GPx) and thioredoxin reductases (TrxR). The main reaction catalyzed by GPx is reduction of hydrogen peroxide to water and glutathione disulfide, while that for TrxR involves reduction of the redox protein thioredoxin, as well as of other endogenous and

exogenous compounds (Lu and Holmgren 2009). Another family of selenoproteins is the deiodinases, which regulate the activity of thyroid hormones e.g. through catalyzation of the conversion from thyroxine (T4) to triiodothyronine (T3, active form; Papp et al. 2007). Many other biological processes have also been linked to selenoproteins, such as biosynthesis of deoxyribonucleic triphosphates, regulation of apoptosis, and immunomodulation (Roman et al. 2014).

2.1.2 Sources and chemical forms

Food items rich in selenium include Brazil nuts, fish, offal, eggs, meat, and cereals (Fairweather-Tait et al. 2011). The amount of selenium in different crops is highly dependent on the selenium content in the soil, and thus, the dietary intake varies widely between different geographical regions (Burk and Levander 2006). Selenium content in the soil largely depends on the type of underlying bedrock, with higher selenium levels in soil derived from e.g. shales, sandstone and limestone (Fairweather-Tait et al. 2011; Hatfield et al. 2016). However, the level of selenium also varies depending on natural (e.g. volcanic and biotic activity) and anthropogenic (e.g. fertilizers) sources.

Areas with unusually high soil selenium include parts of the U.S., Canada, South America, China and Russia (Fairweather-Tait et al. 2011; Hatfield et al. 2016). Such areas may also have elevated levels in drinking water, although in general, intake of selenium from water and air is often negligible. In contrast to these areas, parts of Africa and Australia, New Zeeland, large parts of Europe (including Scandinavia), and other parts of China and Asia, have much lower levels of soil selenium (Fairweather-Tait et al. 2011).

The uptake of selenium in the crops is not only influenced by the total amount in the soil, but by a combination of the chemical form of selenium and the soil conditions (e.g. pH). Selenate (+VI oxidation state) is taken up more rapidly than selenite (+IV oxidation state) in most soil conditions, partly because selenite may form complexes with e.g. iron in acidic soils (Reilly 1996). In the plants, the main form of selenium is selenomethionine, followed by selenocysteine and selenite or selenate (Figure 1). However, in some selenium accumulating plants (e.g. *Astragalus bisulcatus* and *Stanleya pinnata*), the main form of selenium has been found to be methylselenocysteine (Freeman et al. 2006). In meat, the major forms are selenomethionine and selenocysteine, although the ratio between these depend somewhat on the form of added selenium in the feed (Hatfield et al. 2016). These forms are also present in fish, and the selenium concentration has been found to be somewhat higher in marine fish than in freshwater fish (Cappon and Smith 1981). However, it was recently discovered that the major form of selenium in tuna is selenoneine (Yamashita and Yamashita 2010), which was later also found in other types of fish and as a metabolite (Se-methylselenoneine) in human blood and urine (Klein et al. 2011).

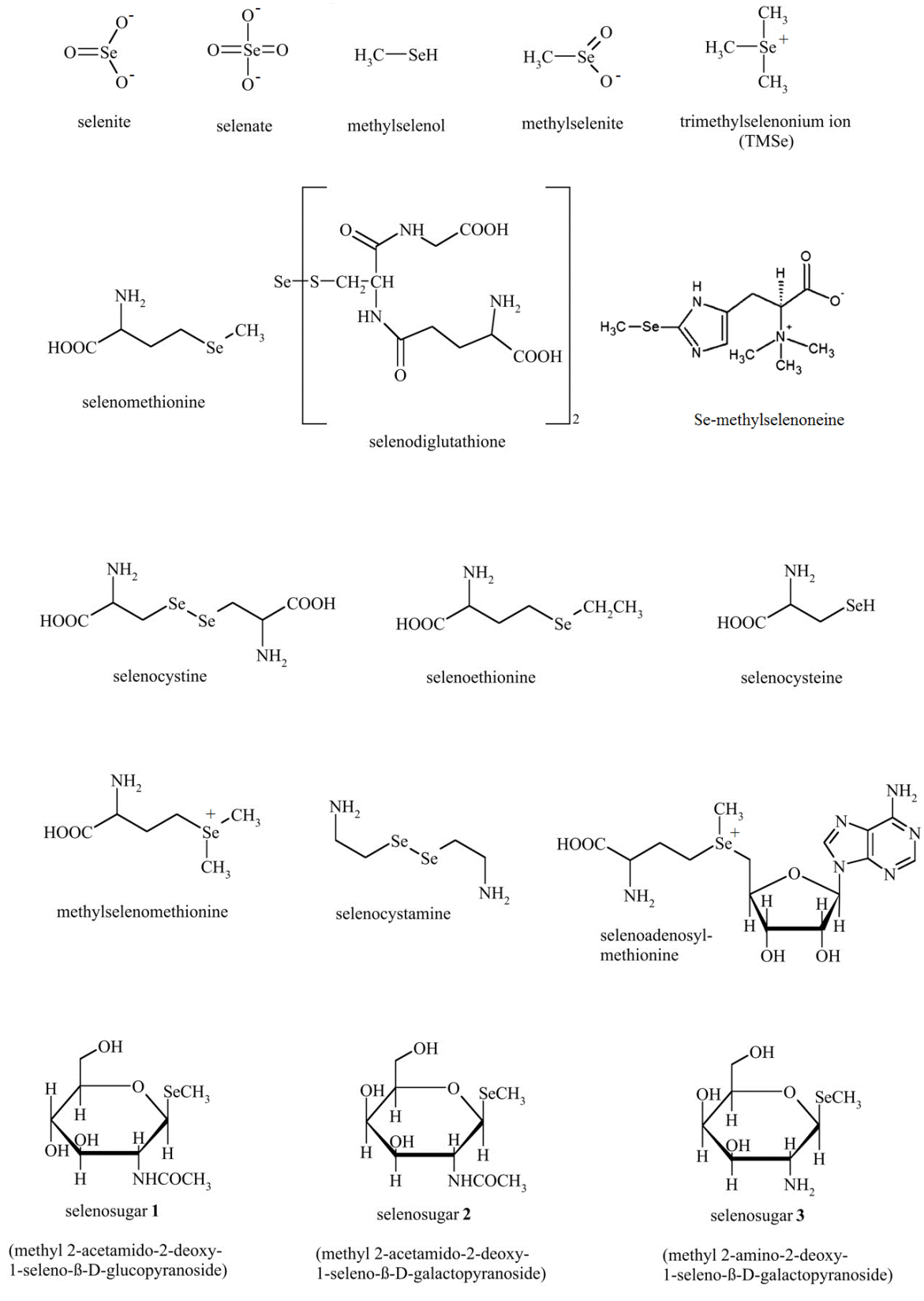

Figure 1. Selenium species claimed to be present in urine of humans or rats. Adapted from (Francesconi and Pannier 2004).

2.1.3 Metabolism

In nutrition, the term “status” refers to a nutrient that is biologically active or potentially active. This means that both the selenium pool that is metabolically functional, as well as the pool that can be readily mobilized to functional forms, are included in the term “selenium status”. Thus, selenium status is the product of intake and absorption, tissue distribution and retention, and metabolism (Combs 2015).

Selenium is generally efficiently absorbed in the lower part of the small intestine. This is believed to occur through multiple membrane transport mechanisms, several shared with those for sulfur (Fairweather-Tait et al. 2011; Roman et al. 2014). There appears to be no homeostatic regulation for the uptake, and the absorption varies between 70-90% of the intake depending on selenium species (Burk and Levander 2006). Once absorbed and transported to the liver, the conversion of dietary selenium (mainly selenomethionine, selenocysteine, selenite and selenate) to selenoproteins involves many intermediate steps for which the details are still unknown. Selenomethionine can be metabolized in a similar manner to methionine, and may be randomly incorporated into methionine-containing proteins (Burk and Levander 2006; Fairweather-Tait et al. 2011). Other selenium species are reduced to selenide, which is incorporated into selenoproteins, transported to other organs, or excreted (Figure 2). The reduction to selenide requires glutathione, and this is a non-enzymatic process. However, the redox potential is too high for selenate, why it must first undergo enzymatic reduction to selenite (Ogra and Anan 2009).

The incorporation of selenium into selenoproteins (in the form of selenocysteine) is dependent on several factors. The UGA codon, usually signaling protein synthesis termination, is also the codon for selenocysteine. However, the incorporation of selenocysteine into the protein requires a selenocysteine insertion sequence (SECIS) that forms a stem-loop structure in the 3’ untranslated region of the mRNA (Berry et al. 1991). In addition to the SECIS-element, tRNA charged with selenocysteine, a selenocysteine elongation factor, selenophosphate synthetase, selenocysteine synthase, and a SECIS-binding protein are also necessary for the selenocysteine biosynthesis and insertion (Kryukov et al. 2003).

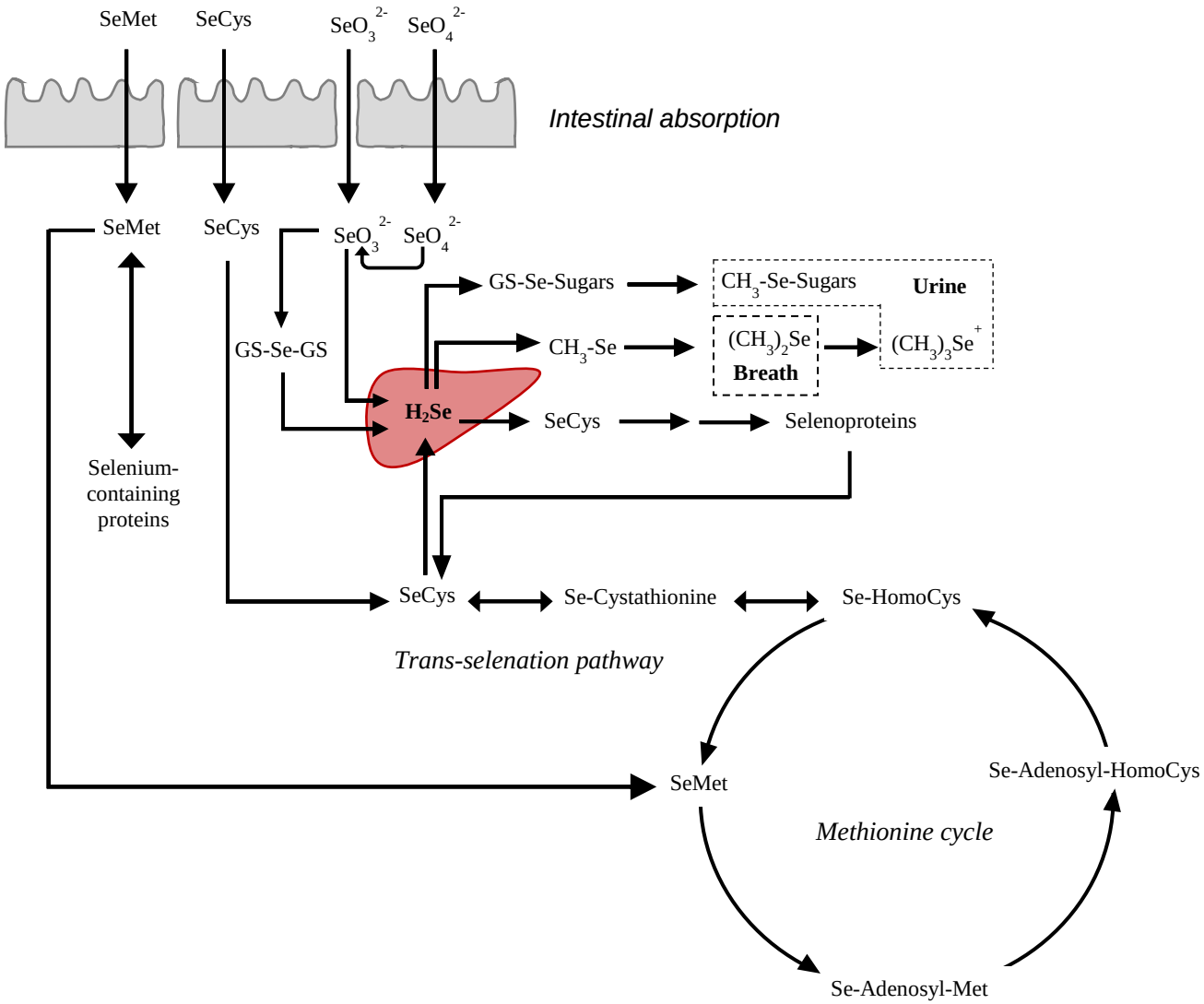

Figure 2. Scheme of human selenium metabolism. Various selenium species are absorbed and transported to the liver for metabolism and production of excretory metabolites as well as selenoproteins, which are then excreted or transported to various tissues, respectively. Adapted from (Fairweather-Tait et al. 2011; Jackson et al. 2013; Roman et al. 2014). Abbreviations: $(Ch_3)_3Se^+$, trimethylselenonium ion; $(CH_3)_2Se$, dimethylselenide; GS-Se-GS, selenodiglutathione; GS-Se-Sugars, glutathione-seleosugars; H_2Se, hydrogen selenide; HomoCys, homocysteine; Se, selenium; SeCys, selenocysteine; SeMet, selenomethionine; SeO_3^{2-}, selenite; SeO_4^{2-}, selenate.

Because of the toxicity of even moderately elevated intake levels [around 850 µg/day for adults (Yang and Zhou 1994)], it is essential for the body to be able to excrete excess selenium. Selenium is methylated via the one-carbon metabolism and excreted mainly in urine, and to some extent breath [mainly at high exposure; Figure 2 (Fairweather-Tait et al. 2011; Jackson et al. 2013; Roman et al. 2014)]. The main forms of selenium in urine are mono-, di-, and trimethylated selenides (Francesconi and Pannier 2004; Suzuki and Ogra 2002), but many other species have been detected in urine either from humans or rats (Figure 1). The monomethylated species include selenosugar 1, 2 and 3 (Francesconi and Pannier 2004). Dimethylselenide is excreted through breath, while the trimethylselenonium ion (TMSe) is a constituent of urinary selenium. It was long believed that TMSe was a major

urinary metabolite only when selenium intake was above nutritional requirements. However, this metabolite is excreted also at normal intake levels, and we recently showed that the production of this metabolite appears to be genetically influenced by polymorphisms in the *INMT* (Indolethylamine N-methyltransferase) gene (Kuehnelt et al. 2015). Formation of TMSe also increased the total urinary selenium excretion. Surprisingly, the prevalence of these polymorphisms was very low in the Argentinean Andes (essentially no producers of this metabolite compared to 1/3 in Bangladesh), despite better selenium status in Argentina than Bangladesh. In the mentioned study, children had lower %TMSe compared to adults, but data is generally lacking regarding potential differences in selenium metabolism between children and adults.

2.1.4 Biomarkers

Methods for determining selenium status have been extensively reviewed (Ashton et al. 2009; Combs 2015; Diplock 1993; Neve 1991; Van Dael and Deelstra 1993), but still, many uncertainties remain, particularly regarding children. The total concentration of selenium in plasma/serum can be determined with good sensitivity. It responds fairly quickly to selenium supplementation or changes in dietary intake, and is therefore the most commonly used biomarker. The biological half-life of selenium differs depending on selenium species, compartment, and dose, although whether this differs between ages is less studied. In general, plasma/serum selenium concentrations are said to reflect intake over the past week or weeks (Neve 1991). However, the concentrations may be influenced by inflammation (Oakes et al. 2008), and also by pregnancy due to the plasma expansion (Faupel-Badger et al. 2007). Concentrations below 40-60 µg/L are often considered deficient based on reduced plasma glutathione peroxidase (GPx3) activity (Fairweather-Tait et al. 2011; Neve 1991). However, the concentration of selenoprotein P saturates at higher plasma concentrations (80-125 µg/L; Burk and Levander 2006; Hurst et al. 2010), and has thus been suggested as a suitable marker for selenium status together with, or instead of, GPx activity and plasma concentration.

Selenium in whole blood, and especially erythrocytes, is considered a more long-term biomarker for selenium status due to the incorporation of selenium during synthesis of these cells and their long life-span (~120 days; Neve 1995; Thomson 2004). Concentrations in erythrocytes are often correlated with those in plasma (Madaric et al. 1994; Stefanowicz et al. 2013), although the response to changes in selenium status is generally slower than that for plasma. About 15% of the erythrocyte selenium is incorporated into glutathione peroxidase 1 (GPx1), and there is a strong correlation between the total selenium concentration in erythrocytes and the GPx1 activity (Stefanowicz et al. 2013). However, the GPx1 activity has been shown to saturate at higher concentrations, while the total erythrocyte selenium concentration still continues to rise with increasing intake. This is because the remaining ~85% of the erythrocyte selenium is bound to hemoglobin (Oster et al. 1988). Due to this binding, it has been suggested that the erythrocyte selenium concentration should be adjusted for the hemoglobin concentration in order to account for variations in hematocrit (Stefanowicz et al. 2013; Vitoux et al. 1999). As concentrations in plasma have been more

widely used to assess selenium status, there is no accepted reference range for selenium concentrations in erythrocytes, either for adults or children.

Other proposed long-term biomarkers of selenium include concentrations in toenails (Longnecker et al. 1996) and hair (Lemire et al. 2009; Yang et al. 1989). However, there is no accepted cut-off for selenium deficiency or toxicity based on concentrations in hair or nails, partly because few studies have compared these concentrations to those in plasma, erythrocytes, or whole blood. In addition, many studies include small sample sizes, and there are also large differences in methods used for hair sampling, washing, and analysis, which may be the underlying reasons for the demonstrated huge variations in measured concentrations of the same hair sample (Seidel et al. 2001). Finally, there are wide variations in reported hair concentrations across studies with normal selenium intake or plasma concentrations (Lemire et al. 2009; Martens et al. 2015; Yang et al. 2010), suggesting that there could also be a difference in tissue distribution depending on unknown factors, although the analytical errors are seldom known.

Several studies have proposed that urinary selenium is a useful biomarker for assessing selenium status since urine is the major route of excretion (Longnecker et al. 1991; Longnecker et al. 1996; Yang et al. 1989), but there are many uncertainties remaining also for this biomarker. Urinary selenium reflects very recent intake, and is therefore considered a short-term marker. Main forms of selenium in urine include the selenosugars (1-3) and TMSe. Besides the genetic influence of *INMT* (Kuehnelt et al. 2015), regulating the formation and excretion of TMSe, there is probably also genetic variation affecting the formation of selenosugars, which also show marked inter-individual variations (Lajin et al. 2016). In addition, it was recently discovered that Se-methylselenoneine was present in the urine of eight volunteers, and that this metabolite constituted 24% of the total urinary selenium for one of the volunteers (<10% for all others; Lajin et al. 2016). Measured selenium concentrations in urine should be adjusted for dilution, or preferentially, measured in 24-h urine samples (Robberecht and Deelstra 1984; Sanz Alaejos and Diaz Romero 1993), although this is not easily achieved in large study groups. Similar to the variation in hair concentrations at comparable plasma/serum concentrations mentioned above, there seem to be marked differences in the ratio between plasma and urine between studies (Table 1).

Table 1. Studies including measurements of both plasma/serum and urinary selenium in healthy children and adults.

Country	Reference	n	Plasma or Serum (µg/L)	Urine (µg/L)	Ratio Plasma/ Urine	Comment
Brazil	Martens et al. 2015	41	107	270	**0.4**	Supplemented children
Brazil	Martens et al. 2015	41	84	40	**2.1**	Non-supplemented children
Turkey	Mengubas et al. 1996	61	42	25	**1.7**	Healthy children
Germany	Jochum et al. 1997	Not stated	69	14.8	**4.7**	Healthy children
Poland	Blazewicz et al. 2015	40	107	58.1	**1.8**	Non-obese children
Czec Rep.	Kvicala et al. 1999	119	59	11.2	**5.3**	Healthy children
China	Lei et al. 2016	35	39	7.74	**5.0**	Children in non-endemic areas
USA	Longnecker et al. 1991	142	198	169	**1.2**	Adults in high Se area

As the interval between selenium deficiency and toxicity is rather narrow, the difficulties in comparing biomarkers between studies raise concern. This is particularly troublesome regarding deficiency and safe intake levels for children, since selenium deficiency may affect child development, and the cut-off for excess intake may be lower than that extrapolated from adults.

2.1.5 Dietary recommendations

During the 1930s, there was an outbreak of a fatal cardiomyopathy in Keshan, China, and it was not until almost 40 years later that it was discovered that the Keshan disease was induced by a very low selenium intake, <12 µg/day (Keshan Disease Research Group 1979). After this discovery, a minimum intake of 20 µg/day in adults was suggested as sufficient to prevent Keshan disease. Still, an intake of 15-40 µg/day (previously reported in New Zealand and Finland) has not been associated with clinical deficiency symptoms, although it is associated with decreased GPx activity. In turn, this may be related to a lower protection against oxidative stress, and a potentially increased risk of cardiovascular disease (Duntas and Benvenga 2015). Therefore, later recommendations of intake were based on optimizing the GPx activity, and the recommendations vary between 30-85 µg/day depending on population category (age groups, pregnancy etc.; Table 2) and country (Rayman 2004).

Table 2. Recommended intake (µg/day) of selenium by population category (Institute of Medicine 2000).

Life stage	Rationale	EAR	RDA	AI	UL
0-6 months	Human milk content			15	45
7-12 months	Human milk + solid food			20	60
1-3 years	Extrapolation from adults	17	20		90
4-8 years	Extrapolation from adults	23	30		150
9-13 years	Extrapolation from adults	35	40		280
14-18 years	Extrapolation from adults	45	55		400
>18 years	Maximizing plasma glutathione peroxidase activity	45	55		400
Pregnancy	Accretion of selenium by fetus + normal requirement	49	60		400
Lactation	Loss of selenium in milk + normal requirement	59	70		400

Abbreviations: AI, adequate intake; EAR, estimated average requirement; RDA, recommended dietary allowance; UL, tolerable upper intake level.

The recommended intake during pregnancy and lactation has been estimated by adding the normal requirement for adults to the amount of selenium acquired by the fetus, and the amount transferred through breast milk. Based on a proposed fetal demand of 4 µg/day, the recommended intake for pregnant women is 60 µg/day. During lactation, the average loss of selenium via breast milk is about 14 µg/day, resulting in a recommended intake of 70 µg/day (Burk and Levander 2006; Institute of Medicine 2000). For infants and children, the recommended intakes have either been extrapolated from adults, or estimated based on concentrations in breast milk (Table 2). Thus, there is a need for more data on the actual requirements for children at different ages.

Symptoms of selenosis (selenium toxicity) in the seleniferous area in China have been observed at intake levels above 850 µg/day (Yang and Zhou 1994). Based on those data, the Institute of Medicine has calculated a tolerable upper intake level (UL) of 400 µg/day for adults (Institute of Medicine 2000), which is slightly higher than that calculated by the European Food Safety Authority (EFSA) of 300 µg/day (European Commision 2000). Thus, the safe range is rather narrow (Figure 3).

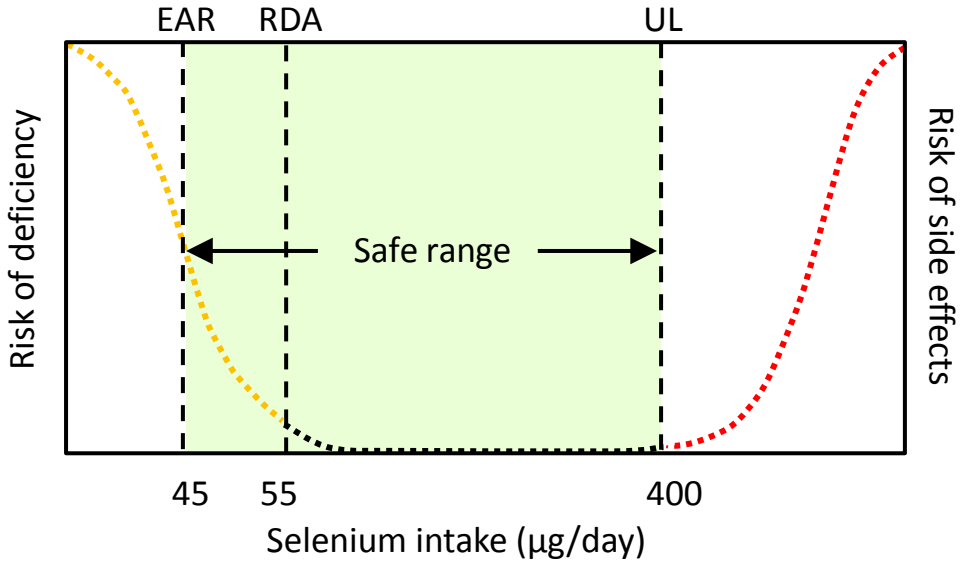

Figure 3. Estimated average requirement (EAR), recommended dietary allowance (RDA) and tolerable upper intake level (UL) of selenium for adults (Institute of Medicine 2000). The narrow interval between essentiality and potential toxicity is marked in green.

The transfer of selenium across the placenta is high, resulting in ratios of maternal/cord blood selenium commonly around 1 (Chen et al. 2014; Rudge et al. 2009). However, the transfer has been shown to differ somewhat depending on selenium species, indicating that specific transfer mechanisms are involved (Santos et al. 2017). The correlation of total selenium in maternal and fetal cord sera in the study by Santos and coworkers was r_S=0.56, $p<0.001$ (n=83), although it was much lower for selenoprotein P (r_S=0.25, $p<0.001$). The average total selenium in cord serum was about 80% of that in maternal serum (56 and 69 µg/L, respectively), while the concentration of selenoprotein P was only 66% of that in maternal serum (28 and 42 µg/L, respectively). Similar serum concentrations of total selenium have been found in Swedish mothers (late pregnancy; 72 µg/L) and newborns (53 µg/L; n=74; Osman et al. 2000). There are no reports of teratogenicity due to maternal selenosis and no overt toxicity in infants with high (not toxic) intakes of selenium through breast milk. Thus, the UL for pregnant and lactating women is the same as for adults in general. For infants, the proposed UL is based on concentrations in breast milk from women in seleniferous areas with no apparent signs of toxicity either in the child or mother (Table 2). As this UL is similar to that for adults per kg body weight, the UL was adjusted to older children based on relative differences in body weight.

However, several recent supplementation studies in adults have found adverse effects (mainly increased risk of cancer and type 2 diabetes) in the groups receiving doses of 200-300 µg/day (Vinceti et al. 2017). Thus, it is likely that the upper limit for safe intake of selenium will be lower in future recommendations. In addition, the assumption that maximized GPx activity would indicate adequate selenium supply has lately been challenged (Vinceti et al. 2017), as there is little evidence that this is actually beneficial to human health. Instead, it has been proposed that part of the increase in enzyme activity with increasing selenium intake is due to compensatory mechanisms, as selenium in high concentrations may also act as a pro-oxidant

(Lee and Jeong 2012). Therefore, Vinceti and coworkers argue that the recommendations set by WHO (i.e. intake of 25-34 µg/day for adults, based on health indicators) should be promoted.

2.1.6 Health effects

Regarding the health effects of selenium deficiency, focus has mainly been on cancer, cardiovascular disease, diabetes, inflammatory disorders, and male fertility, but the results are often conflicting (Fairweather-Tait et al. 2011; Rayman 2012; Vinceti et al. 2014a; Vinceti et al. 2018). Among children, deficiency has mainly been associated with Keshan disease (cardiomyopathy) and Kashin-Beck disease (osteoarthropathy; Ge and Yang 1993).

During pregnancy, low selenium status has been associated with preeclampsia (Xu et al. 2016), and it has also been suggested to play a role in e.g. premature birth, low birth weight, and neural tube defects (Bogden et al. 2006; Mariath et al. 2011; Rayman et al. 2011). However, population-based, prospective studies assessing the importance of selenium during pregnancy for various health outcomes are scarce. Recently, the interest in beneficial effects on cognitive function and neurodegenerative diseases in elderly has increased (Cardoso et al. 2015; Solovyev et al. 2018). As such diseases are often associated with increased oxidative stress (Barnham et al. 2004), it can be hypothesized that selenium could be preventive through its involvement in various antioxidative systems. For the same reason, it may be hypothesized that selenium could be protective in populations highly exposed to pro-oxidants such as arsenic, cadmium, or mercury. Little is, however, known about the importance of adequate selenium status *in utero* or early childhood.

Chronic toxicity following excessive selenium intake (selenosis) has been observed in seleniferous parts of China (Yang and Zhou 1994), with symptoms of toxicity including hair and nail brittleness and loss, gastrointestinal disturbances, rashes, garlic breath odor, fatigue, and irritability. More recent studies have also suggested an increased mortality rate and increased risk of certain types of cancer and type 2 diabetes at intakes even around 200-300 µg/day (Vinceti et al. 2017). However, data regarding adverse health effects in children is lacking.

2.2 THE DEVELOPING BRAIN

2.2.1 Cognitive development

Cognition refers to all inner processes of the mind that result in “knowing”. It includes all mental activity, such as attention, memory, planning and reasoning, problem solving, and creating (Berk 2013). Early cognitive development is a predictor of school progress, and it has been shown that enhancements in education, of girls in particular, result in improvement of child survival, as well as future health, nutrition, and education. Through this chain of events, children without the possibilities to reach their full developmental potential further contribute to the transmission of poverty through generations, thus restricting also future children from reaching their full developmental potential (Grantham-McGregor et al. 2007).

After conception, the nervous system starts to develop very quickly, beginning with the neural tubes (Berk 2013). During the embryonic period (2-8 weeks of gestation), the foundations for all structures of the nervous system are laid (Figure 4). Production of neurons occurs at a very high pace, and at the end of the second trimester, the majority of the millions of neurons are in place and start to differentiate. About half of the brain's volume is made up of glial cells, which support and feed the neurons and are responsible for myelination. These cells multiply rapidly from the 16th week of pregnancy through the second year of life, and the brain weight increases tenfold from the 20th week until birth due to the increase in neural fibers and myelination. The process of cell division slows down through mid-childhood, but accelerates again in adolescence. At birth, brain weight is nearly 30% of the adult brain weight, and increases to 70% at age 2, and 90% at age 6 (Berk 2013).

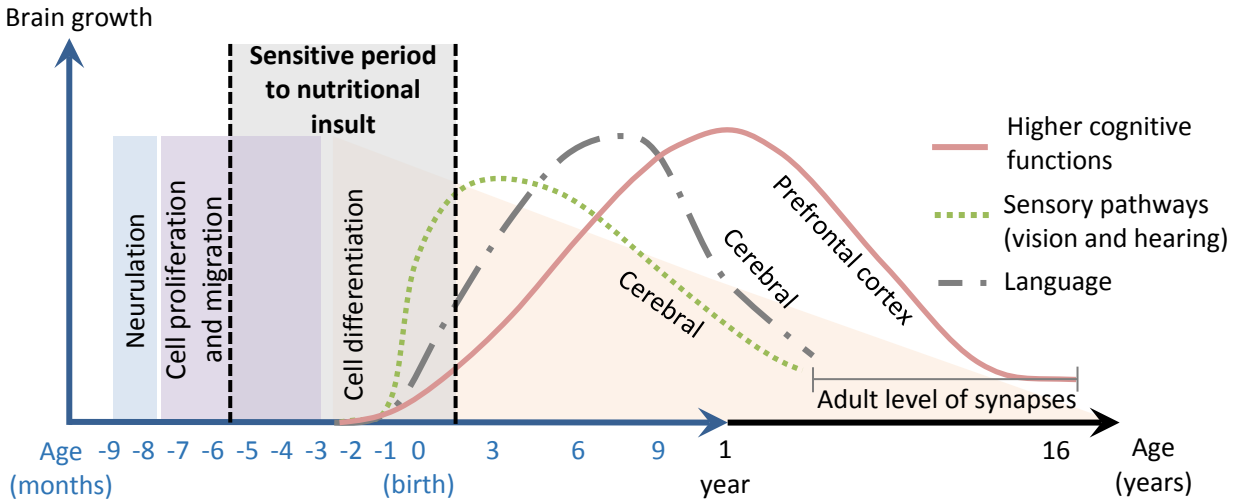

Figure 4. Overview of periods of human brain development, including the period particularly sensitive to nutritional insult (FAO/WHO 2002; Georgieff 2007). Adapted from (Thompson and Nelson 2001).

In the third trimester, the cerebral cortex, the seat of human intelligence, enlarges (Berk 2013). The cerebral cortex surrounds the rest of the brain and accounts for 85% of the brain weight, including a great number of neurons and synapses. This part of the brain is the last to stop growing, and therefore it is also sensitive to environmental influences for a longer time than the rest of the brain.

The order in which different cortical regions develop corresponds to the order of which different capacities emerge in the infant and growing child. The cortical regions with the most extended period of development are the frontal lobes. The prefrontal cortex lies in front of areas controlling movement, and is responsible for consciousness, attention, inhibition of impulses, integration of information, planning, and problem-solving. From the age of two months, the prefrontal cortex is more active. During preschool and school years, it undergoes rapid myelination, formation and pruning of synapses, followed by another period of accelerated growth in adolescence, when it reaches adult levels of synaptic connections (Figure 4). At the rear and the base of the brain is the cerebellum, which is involved in

balance and control of movements. Located in the inner brain is e.g. the hippocampus, which is active in memory and spatial functions. This part of the brain is subject to rapid synapse formation and myelination between the ages of 6-12 months, which is the time when recall memory and independent movement begin.

Even though the prenatal period and the first years of life may be particularly active periods in terms of brain development, regions involved in higher cognition continue to develop into adolescence (Thompson and Nelson 2001). From mid-childhood to adolescence, connectivity among different regions of the cerebral cortex increases (Berk 2013). As a result, adolescents evolve and enhance their cognitive skills such as processing speed, memory, and attention.

Brain development is regulated by nutrients and growth factors both during fetal and early postnatal life. Because of the rapid brain growth towards the end of gestation, the brain is particularly sensitive to nutritional, as well as toxic, insult between 24-44 weeks after conception (Georgieff 2007; Figure 4). Still, early insult may affect the cell proliferation, and thereby cell number (Winick and Rosso 1969), as well as the programming of tissues and functions (Barker 2007). Severe malnutrition during such early periods commonly result in miscarriage or physical birth defects (Berk 2013). Later nutritional insult instead affects differentiation, and thereby size, complexity, and synaptogenesis.

Of particular importance for proper brain development are the thyroid hormones, since they regulate expression of critical neurodevelopmental genes (Rovet 2014). Selenium is an essential component of the iodothyronine deiodinase enzyme that converts thyroxine (T4) to the active hormone triiodothyronine (T3) through removal of one iodine atom. More than 80% of T3 is produced through this conversion in peripheral tissues. Thus, selenium is important for proper thyroid function (Figure 5). In addition, thyrocytes produce H_2O_2, which is then reduced to H_2O by GPx and TrxR in order to protect the thyroid gland from oxidative damage (Duntas and Benvenga 2015; Kohrle 2015).

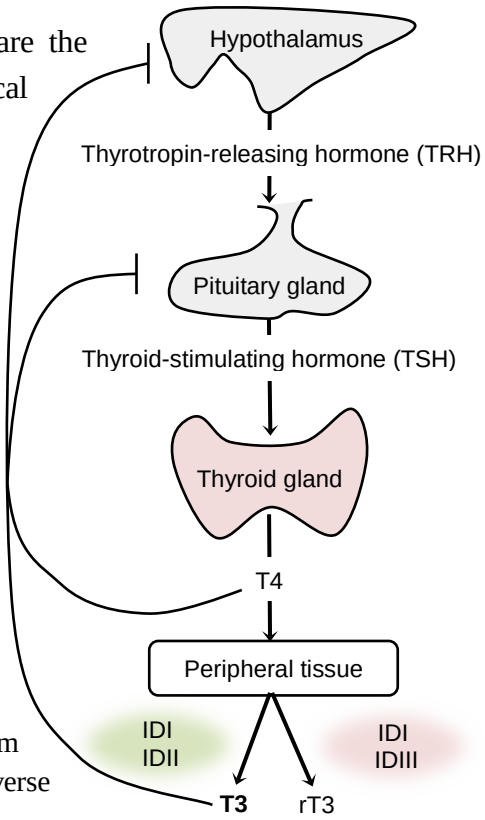

Figure 5. Scheme of the hypothalamic-pituitary-thyroid axis. Selenium is incorporated into iodothyronine deiodinases (ID) I-III, which convert thyroxine (T4) to the active form triiodothyronine (T3; IDI and IDII) or the inactive form reverse triiodothyronine (rT3; IDI and IDIII).

2.3 SELENIUM AND COGNITIVE ABILITIES

Selenium levels in the brain have a tendency to be preserved under conditions of dietary deficiency (Burk et al. 1972), suggesting that selenium is of importance for maintaining brain function.

2.3.1 Experimental studies

In the brain, the highest selenium levels have been found in gray matter areas, especially in the putamen and the pituitary gland (humans), and the cerebellum and cortex (mainly rats; Chen and Berry 2003). Selenium appears to have an important role in the protection of neurons, as selenoprotein P (major contributor to selenium content in the brain) has been shown to enhance neuronal survival and prevent apoptotic cell-death in response to oxidative challenges (Takemoto et al. 2010). For instance, selenoprotein P and other seleno-compounds have been shown to protect motor neurons through interactions with a potent oxidant, peroxynitrite, generated in these cells (Sies and Arteel 2000). Knock-out of the gene encoding selenoprotein P in mice resulted in ataxia (Schomburg et al. 2003), and also in loss of motor co-ordination in mice given a selenium deficient diet (Hill et al. 2003). However, this was not evident among the knock-out mice who received the recommended (or higher) concentrations of dietary selenium, implying that the brain can take up also other forms of selenium. In addition to selenoprotein P, knock-out of deiodinase II (responsible for activation of thyroid hormones) in mice resulted in elevated thyroid-stimulating hormone (TSH) and T4 levels, and a small growth retardation (Schneider et al. 2001). GPx1 knock-out mice exhibited increased sensitivity to neurotoxicants (de Haan et al. 1998), and embryonic knock-down of GPx4 revealed signs of developmental retardation and massive lipid peroxidation (Ufer et al. 2008). The activity of GPx1 seems to be higher in glial cells (mainly astroglial cells, forming the outer layer of the blood brain barrier) compared to neurons (Damier et al. 1993; Power and Blumbergs 2009). This suggests a double-layer protection through selenoproteins formed by e.g. selenoprotein P (inner layer) and GPxs (outer layer; Chen and Berry 2003).

In addition to motor functions, selenium compounds have been reported to enhance the cognitive abilities tested by the object recognition test, the Y-maze test, and the Morris water maze tests in rodents (Rosa et al. 2003; Souza et al. 2010; Stangherlin et al. 2008; Watanabe and Satoh 1995). In the study by Watanabe and Satoh, the selenium-dependent effect was restricted to the female mice. Further animal studies in rodents fed a low-selenium diet have shown increased sensitivity to drug-induced nigrostriatal degeneration (Kim et al. 2000), and a preventive effect of selenium supplementation on dopamine loss, degeneration of neurons, and lipid peroxidation (al-Deeb et al. 1995; Imam et al. 1999; Zafar et al. 2003). Moreover, administration of selenite to rodents has resulted in improved cognitive scores and reduced neurodegeneration (van Eersel et al. 2010).

Selenium supplementation during gestation has been associated with increased concentrations of selenium in the brain of the subsequently born pups (Bou-Resli et al. 2002), who also had increased levels of T3 and T4. In addition, prenatal selenium supplementation was associated

with a reduction in anxiety-like behavior in young pups, and increased cognitive functions in adulthood (Laureano-Melo et al. 2015). However, at toxic doses, selenium supplementation during gestation has been associated with impaired learning and motor function in the subsequently born pups (Ajarem et al. 2011).

2.3.2 Adults and elderly

Since selenoproteins appear to have important functions for neurons, astrocytes, and microglia, decreased expression could be associated with cognitive decline also in humans. Indeed, poor selenium status, assessed as plasma, erythrocyte, or nail concentrations, has in several cross-sectional studies been associated with cognitive decline (Gao et al. 2007; Rita Cardoso et al. 2014) and reduced motor function and muscle strength in elderly (Beck et al. 2007; Lauretani et al. 2007; Shahar et al. 2010). However, the study by Rita Cardoso and coworkers consisted of a small sample (27 Alzheimer patients, 31 elderly with mild cognitive impairment, and 28 controls) and merely compared plasma selenium concentrations across the groups. The study by Gao and coworkers used selenium concentrations in nails (long-term marker; n=2000) of lifelong residents of the same town or village. Therefore, the authors suggest that the exposure reflected lifelong selenium status.

A 9-year longitudinal study (n=1389) also showed an increased probability of cognitive decline in elderly who had a marked decrease in plasma selenium concentration over time (Akbaraly et al. 2007). This study concluded that selenium status decreases with age, and that this may contribute to the decline in neuropsychological functions among aging people, although the possibility of parallel events cannot be excluded. In the same cohort, it was also shown that low baseline plasma selenium, as well as elevated levels of oxidative stress, were associated with an increased risk for cognitive decline, and that the association between oxidative stress and cognitive decline was stronger in the group with lower selenium status (Berr et al. 2000). In addition, supplementation with one Brazil nut per day [can contain 3-36 µg selenium/g nutmeat (Chang et al. 1995)] for six months has been shown to increase selenium levels as well as cognitive functions among elderly with mild cognitive impairment in a randomized control trial (Rita Cardoso et al. 2015). However, studies on associations between selenium concentrations in different tissues or blood fractions and Alzheimer's or Parkinson's disease have been inconclusive (Chen and Berry 2003; Solovyev et al. 2018).

Better selenium status in adults (measured in plasma) has also been positively associated with motor function (Lemire et al. 2011), although another study found no correlation between selenium status and performance on neuropsychological tests (Kunert et al. 2004). In addition to poor selenium status, selenium intoxication has been associated with signs of motor and sensory abnormalities (Vinceti et al. 2014b; Yang et al. 1983).

2.3.3 Early life

Little is known about the importance of adequate selenium status for fetal and child development, especially for the development of the brain, which continues well into adolescence.

The first clinical reports linking poor selenium status and neurological disorders in children suggested that a type of infant seizure was associated with low selenium status, and this condition could in fact be treated with selenium supplementation (Ramaekers et al. 1994; Weber et al. 1991). However, until the work in this thesis, there were only two small cross-sectional studies regarding the association between selenium and cognitive function in children. In a Bangladeshi study of arsenic exposure and motor function among 8-11-year-old children (selected based on low or high concentrations of arsenic and manganese in drinking water), low concentrations of selenium in blood were associated with poor motor function in one of the sub-tests (Parvez et al. 2011; n=304). No beneficial effect of selenium on IQ was reported in a study on Inuit preschool children, designed to evaluate potential adverse effects of lead, mercury, and polychlorinated biphenyls (PCBs; Despres et al. 2005; n=110). However, the blood selenium concentrations in the Inuit cohort were exceptionally high [average concentration corresponding to intake above the UL of 150 µg/d for 4-8 year old children (Institute of Medicine 2000)]. In addition, the aim of the study was not to assess selenium, this was merely included as a covariate for which the estimate was not reported. In an additional study on the same study population, the authors reported an inverse association between child blood selenium and latency of visual evoked potentials (Saint-Amour et al. 2006), although the analyses included only 72 children. Even the lowest blood selenium concentration was high (158 µg/L), implying that a non-linear association would probably have been impossible to observe.

Besides the few studies on concurrent selenium status and children's cognitive abilities, there was one Chinese study that assessed the impact of selenium status on early postnatal neurodevelopment. The authors found a positive association between cord serum selenium concentrations and scoring on the Neonatal Behavioral Assessment Scale at 3 days of age (NBNA; test of neurodevelopment) at concentrations up to 100 µg/L (n=927; Yang et al. 2013). However, at higher concentrations, the association turned inverse (n=80). Thus, prior studies on this topic are very limited.

2.4 INTERACTIONS BETWEEN SELENIUM AND TOXIC METALS

It has been suggested that selenium could protect against the toxic effects of metals/metalloids such as arsenic, cadmium, and mercury. The toxicity caused by such elements occurs largely through generation of reactive oxygen species (ROS; Valko et al. 2005), and the suggested mechanisms for the protective effect of selenium include antioxidative protection from selenoproteins such as GPx and TrxR. Also sequestration into inert conjugates to be excreted has been proposed. Even though such complex formation could potentially contribute to lower metal-induced toxicity, this could also result in functional selenium deficiency.

2.4.1 Arsenic

Arsenic is a toxic metalloid with severe health effects such as cancer, cardiovascular diseases, liver and kidney disease, and diabetes mellitus (Abdul et al. 2015). Human exposure includes

two main chemical forms of arsenic: inorganic (iAs; e.g. arsenite and arsenate) and more complex organic compounds (e.g. arsenobetaine and arsenosugars). The organic forms are mainly found in seafood, and are considered much less toxic than the inorganic compounds which may be present in drinking water and certain food items, such as rice and algae (EFSA 2009). Rice easily takes up arsenic, as well as several other metals. The level of exposure depends on geological conditions and dietary patterns, and elevated exposure is frequent in areas of Bangladesh, India, China, and Thailand, partly due to elevated concentrations in ground water and partly because of the high rice consumption (IARC 2004). However, elevated well water concentrations may be found also in many other countries.

Once ingested, iAs is methylated through the one-carbon metabolism, with S-Adenosyl methionine (SAM) as the methyl donor, to methylarsonic acid (MMA) and dimethylarsinic acid (DMA; Vahter 2002), which are excreted through urine together with remaining unmethylated iAs. The relative amounts of these arsenicals (%iAs, %MMA, and %DMA) are often used to assess methylation efficiency, which differs largely by individuals and populations due to factors such as genetics [mainly polymorphism in *AS3MT*], sex, age, pregnancy, and dietary factors (Antonelli et al. 2014; Gardner et al. 2012; Li et al. 2008; Lindberg et al. 2008; Pierce et al. 2013; Skröder Löveborn et al. 2016). A more efficient methylation (higher %DMA in urine) has been associated with lower toxicity in adults, compared to those with lower %DMA and higher %MMA. There are also major differences between animal species (Vahter 1999).

An antagonistic relationship between selenium and arsenic was first observed in 1938, when it was discovered that selenium-poisoned rats could be treated with arsenic (Moxon 1938). Later, it was discovered that arsenic and selenium could form a complex (seleno-bis [S-glutathionyl] arsinium ion; $[(GS)_2AsSe]^-$) that has been found in the bile of rabbits and rats (Gailer et al. 2002; Levander 1977), and that has been shown to assemble in erythrocyte lysate *in vitro* (Manley et al. 2006). In these animals, the formation of this complex has been shown to facilitate the excretion of each respective element. Based on these studies, it has been assumed in multiple studies and reports that such a complex is also formed in humans, although in fact, this has never been shown. In addition, the main excretory pathway for both arsenic and selenium in humans is through urine and not bile, why a decrease in toxicity through such a complex is questionable. Besides $[(GS)_2AsSe]^-$, selenite and arsenate have been found to interact directly and form an insoluble selenide complex (As_2Se) in the lysosomes of renal cells (Berry and Galle 1994), but again, this has not been identified in humans.

Since selenium is also methylated through the one-carbon metabolism, this is a potential pathway for interaction between these elements. Indeed, it has been shown that exposure to selenium decreases the arsenic methylation efficiency *in vitro* and in mice (Kenyon et al. 1997; Styblo and Thomas 2001; Walton et al. 2003). However, the few available epidemiological studies are conflicting. A positive association between urinary selenium and arsenic methylation efficiency (%DMA in urine) was observed in two cross-sectional studies

on pregnant women and other adults in Chile and Taiwan, respectively, although neither group reported adjusting the urinary concentrations for variation in dilution (Christian et al. 2006; Hsueh et al. 2003). In the study from Taiwan (Hsueh et al. 2003), the authors did not find any association between serum selenium and the arsenic metabolite pattern in urine, and neither did a study on Bangladeshi adults (n=287; Pilsner et al. 2011) or on pregnant women (Li et al. 2008). Still, the Bangladeshi study reported a positive association between plasma selenium and %DMA in whole blood, which, however, is difficult to determine. We found a positive association between selenium concentrations measured in erythrocytes and %DMA in urine, while the association between urinary selenium and %DMA was inverse, in 488 Bangladeshi children (Skröder Löveborn et al. 2016).

The inverse association between urinary selenium and %DMA could suggest a competition for methyl groups (SAM) and/or glutathione (used for reduction of both elements) between selenium and arsenic (Zeng et al. 2005), which may in turn influence the distribution of each respective element between different biological media. In support of such a competition, we recently found that the production of TMSe was associated with lower %DMA (both metabolites measured in urine) in 223 pregnant women from Bangladesh (Skröder et al. 2018). It should be noted that these elements are not major consumers of methyl groups, especially not during child growth, which requires a considerable expansion of protein and transmethylation products such as creatine and phosphatidylcholine (McBreairty and Bertolo 2016). Still, a decreased ratio of reduced:oxidized glutathione has been associated with lower arsenic methylation efficiency and increased arsenic retention (assessed as increased concentrations in blood) in Bangladeshi folate-deficient adults, i.e. with potentially decreased SAM activity (Niedzwiecki et al. 2014). This implies that arsenic exposure could also increase selenium retention, since the methylation of selenium that precedes excretion is also glutathione-dependent. Yet, the enzyme converting oxidized glutathione to the reduced form is GPx, a selenoprotein of which the expression increases with higher selenium intake, particularly at low to normal intake levels (Whanger et al. 1988). In addition, higher plasma selenium has been associated with a higher ratio of reduced:oxidized glutathione (Galan-Chilet et al. 2014).

Finally, it has also been hypothesized that selenium may interact with cysteine residues on AS3MT (Sun et al. 2014). The modified structure would then inhibit the enzyme activity, which would decrease the arsenic methylation efficiency, resulting in higher %iAs and %MMA, and lower %DMA. We recently found that the association between polymorphisms in *AS3MT* and arsenic methylation efficiency was not present among women who are producers of TMSe, however, we do not know if this was due to inhibition of AS3MT, altered expression of *AS3MT,* or competition for methyl groups or glutathione (Skröder et al. 2018).

To summarize, it is still unclear how arsenic and selenium interact in humans, especially in children. It might occur through several mechanisms depending on e.g. chemical form, dose, or genetics. Also, it is unclear what the health implications of these seemingly complicated

interactions may be. Protective effects of selenium, defined either as high levels in blood or as supplementation, on arsenic-induced skin lesions have been indicated in a few studies (Chen et al. 2007; Yang et al. 2002), while others have found that the risk of arsenic-related skin lesions was not associated with the blood selenium concentration (Chung et al. 2006). Despite a potential protective effect concerning skin lesions, it cannot be taken for granted that selenium protects also against other effects of arsenic.

2.4.2 Cadmium

Cadmium is a toxic metal that we are exposed to mainly through food, in particular cereals, seafood, and offal (Jarup and Akesson 2009). Absorbed cadmium accumulates in the kidney where its half-life is in the order of decades. Therefore, chronic cadmium exposure is associated with health effects such as renal tubular dysfunction. In addition, cadmium has been shown to adversely affect the bone, and exposure is commonly associated with osteoporosis and thereby increased risk of fractures, as well as increased risk of cancer, cardiovascular disease and mortality (Akesson et al. 2014).

Animal studies on interactions between selenium and cadmium have shown an antagonistic relationship between these elements, where selenium can enhance the antioxidant defense system and decrease the oxidative stress caused by cadmium exposure (Zwolak and Zaporowska 2012). Indeed, cadmium has been shown to reduce the GPx activity in tissues including the brain, which could be counteracted by selenium supplementation (Whanger 2001). Such an antagonistic relationship between selenium and cadmium was first observed in 1946, when the authors found a protective effect of injected selenite in animals exposed to a lethal dose of cadmium chloride (Tobias et al. 1946). Later, it was also discovered that injected selenium could be protective against testicular cancer caused by injected cadmium in rats, which was also confirmed by others (Whanger 1985). Still, several of the studies also found an increased concentration of cadmium in testes and blood with increasing selenium. Besides the mechanism of an enhanced oxidative defense, a cadmium-selenium complex with a molar ratio of 1:1 has been shown to form *in vitro*, and this complex was also able to bind to selenoprotein P (Sasakura and Suzuki 1998). However, this has not been shown in humans, and the toxicological importance of such a complex is unknown.

Finally, some observational studies have found a stronger association between cadmium and adverse health outcomes in population strata with the lowest selenium levels (Skröder et al. 2015; Wei et al. 2015), while others have found no clear protective role of selenium against cadmium-induced adverse birth outcomes (Al-Saleh et al. 2014).

2.4.3 Mercury

Mercury is present in the environment in elemental, inorganic, and organic forms (Solan and Lindow 2014). Humans may be exposed to elemental forms though inhalation of mercury vapors from e.g. small-scale goldmining, and from dental amalgam fillings. The most common form of dietary mercury is methylmercury, which is the form of some relevance for this thesis. Human exposure occurs mainly through intake of fish and seafood, in which

methylmercury is bioaccumulated and biomagnified. The main effect of methylmercury is damage to the nervous system (Harada 1995), particularly the developing brain. Exposure to methylmercury during pregnancy has been associated with irreversible effects in children, such as motor and cognitive dysfunctions, even when there are no overt symptoms in the mother (Antunes Dos Santos et al. 2016).

An antagonistic relationship between mercury and selenium was first observed in rats (Parizek and Ostadalova 1967), where co-administration of sodium selenite prevented the nephrotoxic effects of inorganic mercury. Co-exposure to selenium (often high doses) has also been shown to reduce the acute toxicity of both inorganic mercury and methylmercury, as well as to prolong the life expectancy, growth rate, and decrease the neurotoxicity in experimental studies (Cuvinaralar and Furness 1991). Several studies have found that selenium decreases the retention of mercury (both inorganic and organic) in kidneys, while others have found that selenium increases the methylmercury concentrations in e.g. the brain (Whanger 2001). Given these contrasting findings, it has been suggested that there might be different mechanisms involved in the interactions depending on the chemical forms of both elements (Cuvinaralar and Furness 1991). These mechanisms include *i)* redistribution of both elements between tissues, *ii)* competition for binding sites, *iii)* complex formation into inert, non-toxic conjugates, *iv)* conversion from toxic forms to less or non-toxic forms, and *v)* prevention of oxidative damage. Indeed, it has been reported in *in vitro* studies that mercury may inhibit both GPx (Franco et al. 2009; Hirota et al. 1980) and TrxR (Carvalho et al. 2008).

However, there are few human studies that have assessed a potential protective effect of selenium on methylmercury toxicity. A recent epidemiological study from the Faroe Islands did not find evidence of an interaction between cord blood mercury (mainly methylmercury) and selenium when assessing children's neurological function at 7 years (Choi et al. 2008). However, there was an indication of a stronger adverse mercury-related effect when assessing finger tapping at 7 years in the lowest group of cord blood selenium. In addition, the authors suggest that a protective effect of selenium was not detectable due to the high relative concentration of selenium to mercury (molar ratio), implying that children in the lowest selenium group were also protected.

3 AIMS

The overall aim of the present thesis was to clarify the impact of early-life selenium status on child development. Specifically, this PhD thesis aimed to elucidate:

- The influence of maternal selenium status during pregnancy on children's cognitive abilities at 1.5 years of age (**Paper I**),

- If hair is a useful biomarker for assessment of selenium status, as well as internal dose of other elements, in the studied children (**Paper II**),

- Whether any effetcs of maternal selenium status during pregnancy on children's cognitive abilities are still present when children are older, and whether children's own selenium status is also of importance (**Paper III**),

- Factors influencing selenium status and kinetics in children (**Paper IV**).

4 MATERIALS, METHODS, AND CONSIDERATIONS

4.1 STUDY AREA AND PARTICIPANTS

4.1.1 MINIMat

The studies included in the present thesis are based on data from a mother-child cohort nested in a food and micronutrient supplementation trial established in Matlab, a rural area 53 km south-east of Dhaka, Bangladesh (Figure 6).

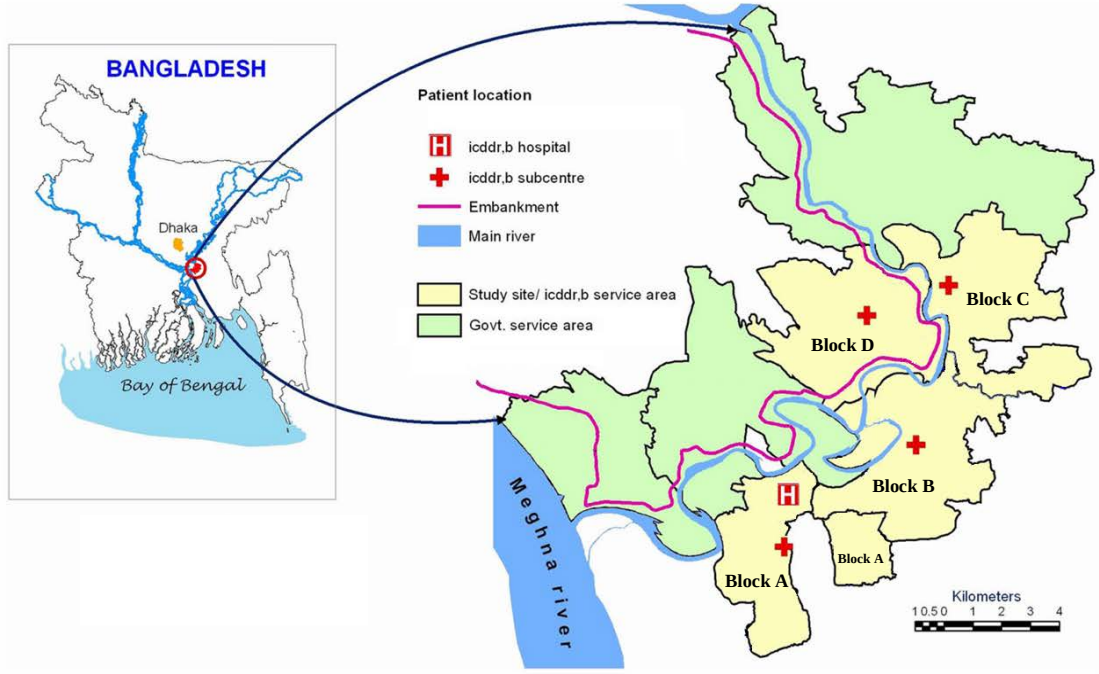

Figure 6. Map of Matlab, Bangladesh, including the health care facilities (sub-centers) and hospital. Adapted from (Nasreen et al. 2014).

In this area, the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b), provides health services via a central hospital and four health care facilities. In addition, icddr,b has been collecting information via a health and demographic surveillance system (HDSS) through monthly visits by experienced community health workers. Information such as pregnancies, births, deaths and migration has been registered over the past 60 years (Karar Zunaid Ahsan and Rahman 2009).

The original study (MINIMat, the Maternal and Infant Nutrition Intervention, Matlab) was a food and micronutrient supplementation trial during pregnancy, implemented from October 2001 to November 2003 (Persson et al. 2012). Eligibility criteria for enrollment were viable fetus, gestational age less than 14 weeks, no severe illness, and written consent for participation. In total, 4436 pregnant women were recruited and randomized into three supplementation groups [starting from gestational week (GW) 14]; either 30 or 60 mg iron and 400 µg folic acid (the latter WHO's standard supplementation for pregnant women) or a

multiple micronutrient capsule containing 30 mg iron and 400 µg folic acid together with 13 additional micronutrients, including 65 µg of selenium in the form of sodium selenite. This daily supplement was combined with either early (GW9) or usual (GW20) start of food supplementation (Persson et al. 2012), resulting in six groups. The food supplementation consisted of 80 g of roasted rice powder, 40 g of roasted pulse powder, 20 g of molasses, and 12 mL (6 g) of soybean oil, which provided a total of 608 kcal. The food supplementation was provided six days a week. Out of the 4436 pregnant women recruited to the trial, 845 were lost-to follow up during pregnancy due to fetal loss (10%), out-migration from the study area (4%), or refusal to participate (3%). In total, there were 3625 live births, and 3267 were singletons with anthropometry measured at birth.

Early in the study it was discovered that elevated exposure to arsenic through drinking water was common in this area, and therefore a longitudinal research project was initiated to evaluate potential developmental effects of arsenic and other environmental pollutants, mainly dietary toxicants (see e.g. Gardner et al. 2013; Kippler et al. 2012b; Vahter et al. 2006). For exposure assessment in early pregnancy, we kept an aliquot of the urine sample collected for pregnancy testing. The present thesis is based on this nested cohort of pregnant women and their subsequently born children who have been followed-up for growth, immune function and toxicity markers, as well as developmental assessment until 10 years of age.

4.2 DESIGN

For the follow-up of child health in relation to toxic and essential elements, the children were divided in two groups (immune cohort and developmental cohort) in order to limit the study burden for each child. The studies in the present thesis (**paper I-III**) are based on both groups. For a brief overview of the studies, see Figure 7.

Out of the 3267 single births in MINIMat, 2853 mother-child pairs with children born between May 2002 and December 2003 were invited to participate in the developmental follow-up when the children were 1.5 years, and 2112 agreed to participate (74%). The main reasons for loss to follow-up were being away from home on visits (n=351, 47%), refusal (n=185, 25%), death (n=89, 12%), moving out of the study area (n=52, 7%), disability (n=5, <1%) and illness at the time of testing (n=59, 8%; Hamadani et al. 2010b). Of the mothers recruited throughout 2002 (n=2119), 900 had donated blood samples in late pregnancy (GW30) that were analyzed in 2012-2013 for other purposes (Rahman et al. 2015). Reasons for missing blood samples were blood samples being used for other purposes and refusal. The overlap between these two groups (children born May 2002-December 2003 and women recruited throughout 2002) constituted the sample used in **paper I** (n=750).

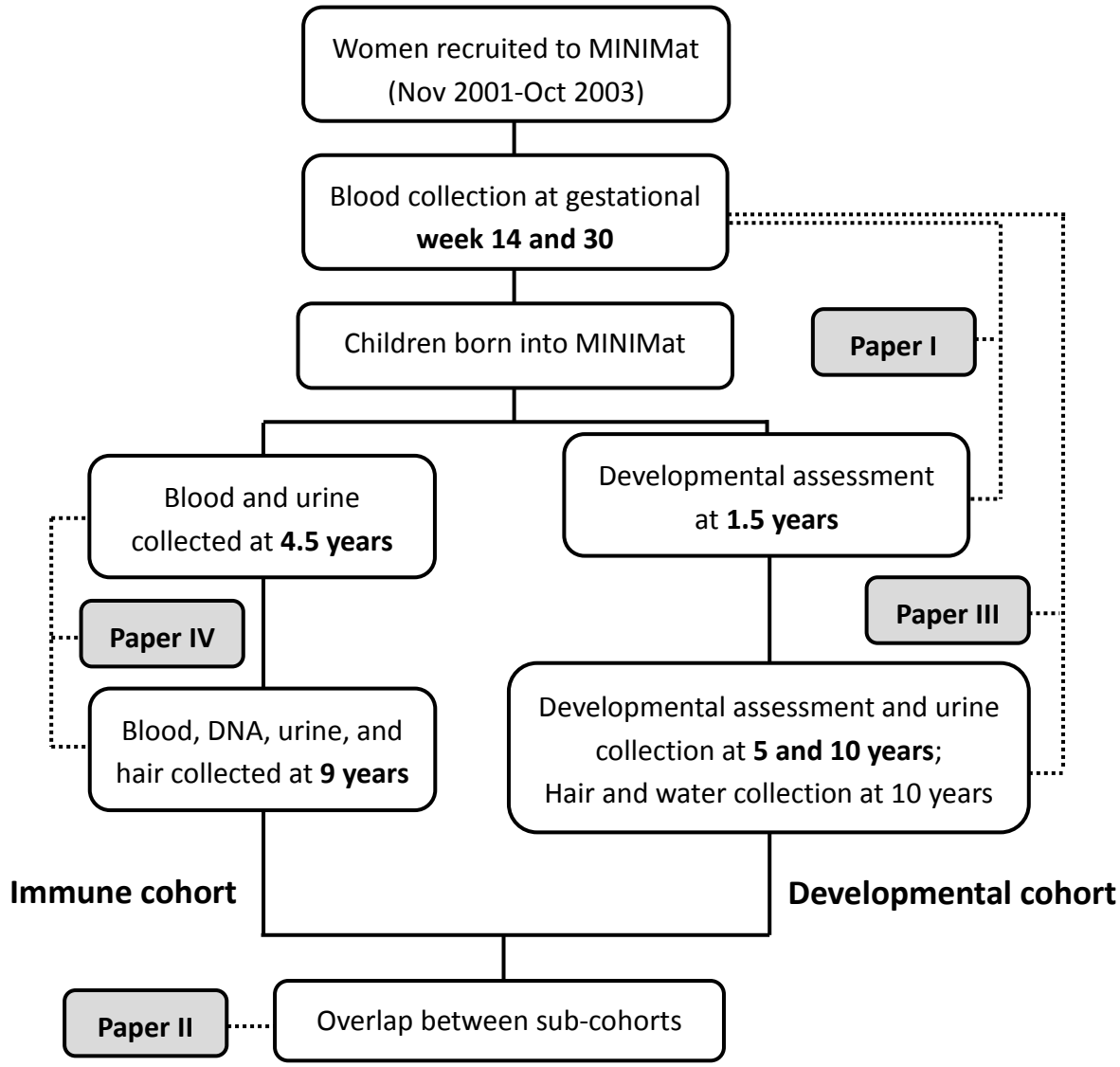

Figure 7. Project overview for **paper I-IV** and the division of the MINIMat children into the two groups for assessment of either immune function (immune cohort, left) or cognitive development (developmental cohort, right).

At the developmental assessment at 5 years, 2260 of the children participated (79% of invited), and finally, children born between October 2002 and November 2003, who participated in the follow-ups at both 1.5 and 5 years (n=1885), and were alive and registered as residents in the study area, were invited to the follow-up at 10 years (n=1607). Out of these children, 1530 (95%) agreed to participate. We had available blood samples from GW14 for more women than we had from GW30, and after the findings in **paper I**, we chose to analyze and use the blood samples from GW14 to increase the sample size for **paper III**. In total, blood from GW14 was available from 1408 women, and this group of mother-child pairs constituted the sample for **paper III**.

Paper II and IV were based on children in the immune cohort (Figure 7). Children born from June 2003-June 2004 were followed-up at 4.5 and 9 years for assessment of especially immunotoxic effects of arsenic, and these children donated blood, urine, and at 9 years also hair. There was an overlap between these two sub-cohorts consisting of children born

between June and December 2003, for whom all biological samples were collected (in the immune cohort), as well as water and hair samples (in the developmental cohort) at 10 years of age. These children, who participated in both sub-cohorts, constituted the sample used in **paper II** (n=207).

Blood and/or urine samples from 511 9-year-old children were available for analyses of toxic metals and essential elements (immune cohort). Out of these children, 395 had enough high-quality DNA for genotyping, and all covariates available. Thus, these 395 children constituted the sample used for **paper IV**, which also included 259 children from the immune cohort with available samples (erythrocytes and urine) from 4.5 years for comparison (243 of which overlapped with the 395).

4.3 SAMPLING AND DATA COLLECTION

4.3.1 Biomarkers of exposure and status

As discussed under section 2.1.4, there are great uncertainties regarding suitable markers of selenium status, especially in children. Therefore, we have put much emphasis on the evaluation of selenium concentrations in the different biological media available in the study cohorts and factors influencing these concentrations. This felt important in order to evaluate the impact of selenium status on child development.

Maternal selenium status was evaluated through concentrations in erythrocytes (**papers I** and **III**), and concentrations in urine were also measured. For a sub-sample, we also had plasma selenium for comparison (n=98). For children in the developmental cohort, we measured selenium in urine at 5 and 10 years, and also in hair at 10 year (**paper III**). At 4.5 and 9 years, selenium was measured in erythrocytes and urine, and at 9 years also in plasma and hair (**papers II** and **IV**). We calculated the ratio between plasma/urinary selenium at 9 years as an estimation of selenium retention, where a higher ratio indicates higher retention. We also calculated the ratio between erythrocyte/plasma selenium to assess distributional changes between the blood compartments in relation to the assessed factors (**paper IV**).

Community health workers visited Matlab families monthly on a routine basis, and women who reported that they had missed their last menstruation were tested for pregnancy by urine test. In case of positive results, about 20 mL of the urine collected in plastic urine collection cups was transferred and kept in a 24 mL plastic, acid-washed, bottle for long-term storage. This sample was collected at GW8 on average. During the antenatal visits at GW14, 19, and 30, and at the follow-ups of the children at 4.5 and 9 years (immune cohort), or 5 and 10 years (developmental cohort), spot urine samples were collected at the health care facilities. All samples were refrigerated until they were frozen at -70^{o}C at the Matlab hospital.

During pregnancy, blood samples were collected in 5.5 mL Li-heparin tubes (Sarstedt, Nümbrecht, Germany) when the women visited the health care facilities at approximately GW14 and 30. From the children that participated in the immune cohort, venous blood samples were collected in Na-heparin tubes (Vacuette, Greiner Bio-One International AG,

Kremsmünster, Austria) at 4.5 and 9 years of age. Samples were transported to the Matlab hospital and centrifuged for separation of plasma and erythrocytes, and the fractions were stored at -70 and -20^{o}C, respectively. The sampling material used for both blood and urine collection had been tested to be trace element free.

For the follow-ups at 9 and 10 years, the community health workers were trained (orally and by detailed written instructions) to collect hair samples at the local health care facilities. Hair samples from the occipital part of the children's heads were cut with 18/8 stainless steel scissors, as close to the scalp as possible. The sample was tied together with a nylon thread (less contaminants compared to cotton) at the end closest to the scalp, and placed in high quality paper envelopes to avoid static electricity.

At the follow-up at 9 years, children participating in the immune cohort were sampled before and after a vaccination, with 21 days in between (Raqib et al. 2017). Because the samples were also used for other purposes, the samples included in **Paper IV** were from different time points. For plasma and urine, we had samples collected before vaccination, while the erythrocytes were from the second sampling. For hair, the samples used in **paper II** were those collected in the developmental cohort (9.5-10 years of age, at the time of water collection), and these samples were approximately half of those included in **paper IV**. The remaining hair samples used in **paper IV** were collected in the immune cohort, prior to the vaccination (9 years).

All samples were transported frozen and analyzed at Karolinska Institutet, Sweden, except for hair samples which were kept and transported at room temperature.

4.3.2 Covariates

Information on maternal characteristics was obtained either at enrollment into MINIMat [maternal age, BMI (kg/m^2), education, parity] or from HDSS (e.g. assets and housing conditions). Family socioeconomic status (SES) was derived using a wealth index based on factors such as lands, assets, housing structure, and household sanitation (Gwatkin et al. 2000). This information was updated at the follow-ups of the children, and so was the information regarding maternal and paternal education (years of formal schooling). The SES score was used both as a continuous variable (**papers I-III**), but was also divided into quintiles for ease of interpretation (**paper IV**). Maternal non-verbal IQ was assessed at the 5-year follow-up using the Raven's Coloured and Progressive Matrices. In addition, the quality and quantity of children's stimulation at home was assessed at both 1.5, 5, and 10 years using a modified version of Home Observation for Measurement of the Environment (HOME; Bradley et al. 2003; Caldwell 1967), which is based on questions regarding e.g. responsibility, parent-child interactions, learning materials and opportunities, family integration, and physical environment.

Children's birth anthropometry was measured according to standard procedure by the attending nurse or by a trained paramedic for those with home delivery (Persson et al. 2012). Birth weight was measured using an electronic beam scale precise to 10 g. Children's height

was also measured at each follow-up using either a locally manufactured wooden stadiometer (precise to 0.1 cm; used for developmental cohort) or a free standing stadiometer (Seca 214, Leicester Height Measure; Seca GmbH & Co., Hamburg, Germany; used for immune cohort). Children's weight was measured using a digital scale (TANITA HD – 318, Tanita Corporation, Japan) precise to 0.1 kg at all follow-ups. In addition to their height and weight, BMI was calculated, as well as standardized scores (HAZ, height-for-age z-score; WAZ, weight-for-age z-score; BAZ, BMI-for-age z-score) using the WHO growth references (WHO 2010). Underweight was defined as WAZ<−2 (lower than two SDs from the median weight-for-age of the reference population defined by WHO) and stunting was defined as HAZ<−2. Children's hemoglobin (Hb) was measured at 4.5, 9, and 10 years in peripheral blood (finger prick), using a HemoCue photometer (Hemocue AB, Ängelholm, Sweden).

At the follow-up at 5 years, information on the type of school [none, primary, Madrasa (Islamic), kindergarten, Maktab or non-formal] was collected, and this information was updated at the follow-up at 10 years [none, public primary school, Madrasa school, NGO (nonprofit private) school, and English medium (private school)]. In addition, information on children's years of formal education was collected at the 10-year follow-up. Season of sampling was defined as pre-monsoon (January-May), monsoon (June-September), or post-monsoon (October-December).

At 9 years, children were genotyped for *INMT* using allelic discrimination for SNPs rs6970396, rs4720015, and rs1061644. Samples were analyzed on a LightCycler 480 II with software SW 1.5.1 (Roche, Switzerland) using TaqMan probes (Assay ID C__29402264_10, C__25755281_30, and C__2641592_10; Applied Biosystems). No SNPs deviated from Hardy-Weinberg equilibrium, which was evaluated using chi-square analysis [criterion for deviations $\chi^2>3.84$ ($p<0.05$)]. Data is presented only for rs6970396 (**paper IV**), as the three SNPs were in complete linkage disequilibrium (Kuehnelt et al. 2015).

Finally, arsenic, cadmium, lead, manganese, and zinc were analyzed in erythrocytes and urine, and mercury and arsenic were analyzed also in hair, and arsenic also in plasma, as described in section 4.4.1.

4.4 ANALYTICAL METHODS

4.4.1 ICP-MS

We measured the concentrations of selenium, as well as multiple, other elements, in all samples using inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7700x, Agilent Technologies, Tokyo, Japan) equipped with octopole reaction system collision/reaction cell technology, at the Unit of Metals and Health, Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden. With this method, aerosol droplets of liquid samples are introduced into a torch with ionized argon gas, in which molecules are atomized and ionized (positively). The ions are then directed into the mass spectrometer, where they are separated based on their mass-to-charge ratio, and translated to

an electric signal that can be measured and quantified based on calibration standards. This technique provides a lower detection limit and requires a smaller sample volume, offers higher throughput, and is applicable to more elements compared to other techniques such as atomic absorption (Nageswara Rao and Talluri 2007). Selenium (and calcium) was analyzed with the collision/reaction cell in hydrogen gas mode, while other elements were analyzed in helium mode (magnesium, manganese, iron, cobalt, copper, zinc, arsenic, molybdenum, and cadmium) or in no gas mode (lead, mercury, and iodine) to minimize spectral interferences.

4.4.2 Sample preparation

4.4.2.1 Urine

Prior to analyses, urine samples were diluted 1:10 in 1% nitric acid (65% w/w, Scharlau Trace Analysis Grade, Scharlab S.L., Sentmenat, Spain). Concentrations in urine were adjusted to the average specific gravity (1.012) measured by a digital refractometer (RD 712 Clinical Refractometer, EUROMEX microscopes, Holland) to compensate for variations in dilution (Nermell et al. 2008). This method provides concentrations of total selenium in urine, which was also measured for cadmium. For arsenic, however, total urinary concentrations include also organic species (considered less toxic than the inorganic species). Therefore, urinary arsenic was speciated using high-performance liquid chromatography on-line coupling with hydride generation ICP-MS (HPLC-HG-ICP-MS), as described before (Gardner et al. 2011). The sum of the measured arsenic species [iAs (III), iAs (V), MMA (V), and DMA (V)] was then calculated and used as a measure of exposure to iAs. For iodine, samples were diluted 1:10 with 0.1% NH4OH and analyzed in no gas mode, as described in detail before (Rydbeck et al. 2014).

4.4.2.2 Blood fractions

For erythrocyte fractions from GW14 analyzed in 2007, as well as all those collected at GW30 (analyzed in 2012-2013), sample preparation included acid digestion which has been described in detail previously (Kippler et al. 2009). In short, about 0.5 g of sample was digested in quartz tubes in 2 mL concentrated HNO_3 (65% suprapur, Merck, Darmstadt, Germany) and 3 mL deionized water under high pressure (40 bar) and temperature (250^{o}C) for 30 minutes in a Milestone ultraCLAVE II microwave digestion system (EMLS, Leutkirch, Germany). Digested solutions were then diluted to a final acid concentration of 20%. The remaining blood samples (erythrocyte fractions from GW14, 4.5, and 9 years, and plasma fraction from 9 years) were diluted with an alkaline solution, which after careful testing had shown comparable results with the digestion method and even lower LOD for selenium (Lu et al. 2015). About 0.2 mL of sample was diluted 1:25 in a solution containing 2% (wt:vol)1-butanol, 0.05% (wt:vol) EDTA, 0.05% (wt:vol) Triton X-100, 1% (wt:vol) NH_4OH and 20 µg/L internal standard (Sigma-Aldrich, Schnelldorf, Germany), after which the samples where vortex mixed, sonicated for 5 minutes, and centrifuged at 1000 rpm for 2 minutes [MSE centrifuge, Super Minor, MSE (UK) Ltd, London, England]. We also adjusted the erythrocyte concentrations for Hb, however the adjusted and unadjusted values were

highly correlated (**paper I**). Plasma samples (used in **paper IV**) were also diluted in the alkaline solution described above, although the dilution factor was 1:60 due to very small sample volume remaining. To assess the impact of such a high dilution factor, we first performed a pilot study to assess whether this had any impact on the quantifiability. We measured selenium and arsenic in plasma diluted 1:25, 1:50, and 1:60 in 5 different samples (Figure 8).

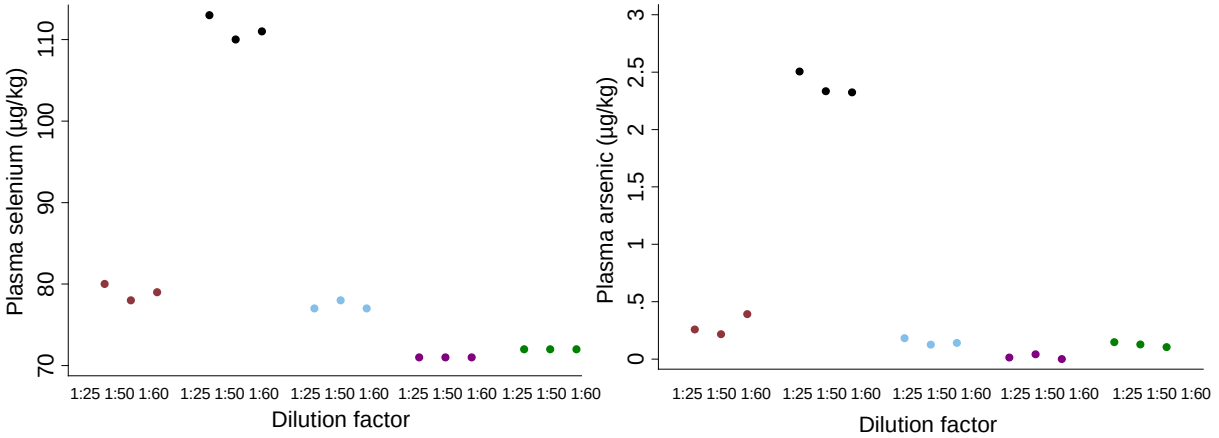

Figure 8. Plasma concentrations of selenium and arsenic in 5 different samples (each color represents one sample) diluted either 1:25, 1:50, or 1:60.

The concentrations differed marginally, and there were no difficulties quantifying the selenium concentration for any dilution factor (signal/noise>3 for all, indicating that the sample signal is well above the signal of the blanks). For arsenic, some samples had a signal/noise ratio<3, suggesting that the lowest concentrations were less precise when using higher dilution factors. However, given the similar results regardless of dilution factor for each respective sample, we did not consider this a major issue. Also, there was no obvious trend for the arsenic concentrations with increasing dilution factor.

In **paper IV**, the element concentrations in the blood fractions were adjusted to the average density of plasma (1.026) and erythrocytes (1.096; Lentner 1981) to convert concentrations from µg/kg to µg/L for comparability with other studies.

4.4.2.3 Hair

Prior to analysis of hair samples, bundles of about 2 cm of hair (about 50 mg; closest to the scalp) were washed in 50 mL 2% Triton X-100 for 1 hour, rinsed 10 times in deionized water, and dried for 24 hours at room temperature. For samples from children with hair shorter than 2 cm, the whole sample was used (minimum 10 mg). The samples were weighed on a calibrated (internal and external) analytical balance (Precisa 202A, Dietikon, Switzerland) close to an alfa source (Staticmaster model 2U500, NRD, US) to avoid static electricity as this may cause large errors in the obtained weight.

In a pilot study, we first attempted to digest the hair samples in concentrated HNO_3 in room temperature overnight, or until the whole sample had dissolved. We then analyzed the

solution after diluting it with deionized water to an acid concentration of 20%. Duplicates of the samples were acid digested as described above for erythrocytes, and the obtained concentrations were compared. However, the concentrations obtained after the alternative method (overnight digestion) did not correspond very well with those from the standard acid digestion method (Table 3), and thus, we chose to digest all samples according to the standard method. For mercury analyses, we diluted the digested hair samples to 20% HNO_3, and also stabilized with 2% concentrated HCl (Emsure®, ACS, ISO, Reag. Ph. Eur., Merck Darmstadt Germany; Gustin et al. 2017).

Table 3. Concentrations of selenium and arsenic (µg/kg) in hair digested by two different methods.

	Ultraclave digestion	Overnight digestion	Ratio
Selenium			
Sample 1	463	649	0.71
Sample 2	451	708	0.64
Sample 3	492	669	0.74
Sample 4	347	515	0.67
Arsenic			
Sample 1	35	47	0.74
Sample 2	37	53	0.70
Sample 3	11	15	0.73
Sample 4	32	44	0.80

4.4.3 Analytical performance

For test of accuracy as well as precision, all analyses described above included commercial reference materials for whole blood, plasma, urine, or hair. For hair, we also included an in-house sample from multiple residents of the Faroe Islands in each analysis. Usually, the reference samples were included in the beginning, middle and end of each analytical run, together with one standard (in the middle of the standard curve). The obtained results were generally in good agreement with the recommended values, as shown in the supplemental material of **paper I-IV,** indicating accurate results. In addition, the variation in the obtained concentrations between all the runs was low, showing high precision. A summary of all reference materials analyzed for selenium together with samples included in any of the studies of the present thesis [except for those already described in (Kippler et al. 2009)] are presented in Table 4.

Table 4. Recommended and obtained concentrations of selenium (µg/L or µg/kg) for all reference materials included in any of the analyses in **paper I-IV**, as well as the limit of detection (LOD; 3xSD of blanks) and coefficient of variation (CV).

	Recommended value	Obtained value	n	CV	LOD
Erythrocytes					
Seronorm Whole Blood 1103129	112 ± 23	119 ± 6.9	137	5.8%	0.025
Seronorm Whole Blood 1103128	59 ± 12	57 ± 3.2	135	5.7%	0.025
Seronorm Whole Blood 1406263	57 ± 11	59 ± 1.3	17	2.2%	0.025
Seronorm Whole Blood 1406264	153 ± 30	162 ± 3.6	18	2.3%	0.025
Plasma					
Seronorm serum Mi0196	83 ± 6	71 ± 5.2	18	7.4%	0.11
Medisafe serum 28341	50 ± 9	50 ± 3.2	18	6.4%	0.11
Urine					
Seronorm urine NO2525	67 ± 7.1	73 ± 4.6	76	6.3%	0.014
Seronorm urine OK 4636	22 ± 2.8	24 ± 2.2	76	9.2%	0.014
NIST2670aH	230 ± 8.3	190 ± 24	36	13%	0.014
NIST2670aL	8 ± 3	7.4 ± 1.1	23	15%	0.014
Seronorm urine 1011645	70 ± 14	74 ± 3.3	54	4.5%	0.014
Seronorm urine 1011644	14 ± 2.8	15 ± 0.51	55	3.4%	0.014
Hair					
NCSZC 81002b	590 ± 40	633 ± 96	30	15%	0.020
IAEA-086	1000 ± 200	1080 ± 80	18	7.4%	0.020
Faroe hair	-	1680 ± 181	112	11%	0.020

The limit of detection (LOD) was calculated as 3xSD of the blanks of each analytical run, and no sample had a selenium concentration below this value for any of the biomarkers used in any of the papers. A few samples had concentrations below the LOD for the other elements analyzed (plasma arsenic in particular, n=6), and we either replaced these with LOD/√2 (if negative, concentrations very close to the blank values), or kept them as measured (if positive).

The two different methods of sample treatment used for erythrocyte analyses were strongly correlated (Lu et al. 2015), although the concentrations from the acid digested samples were consistently about 10% higher than those following alkali dilution. Thus, we adjusted the sample concentrations measured using the acidic method by multiplying them with 0.9 (**paper III**).

To assess whether storage time prior to analysis has any impact on the selenium concentrations, we reanalyzed 20 erythrocyte samples from the children included in **paper IV** after storing them at -80°C for almost four years. The results showed good agreement (Figure 9).

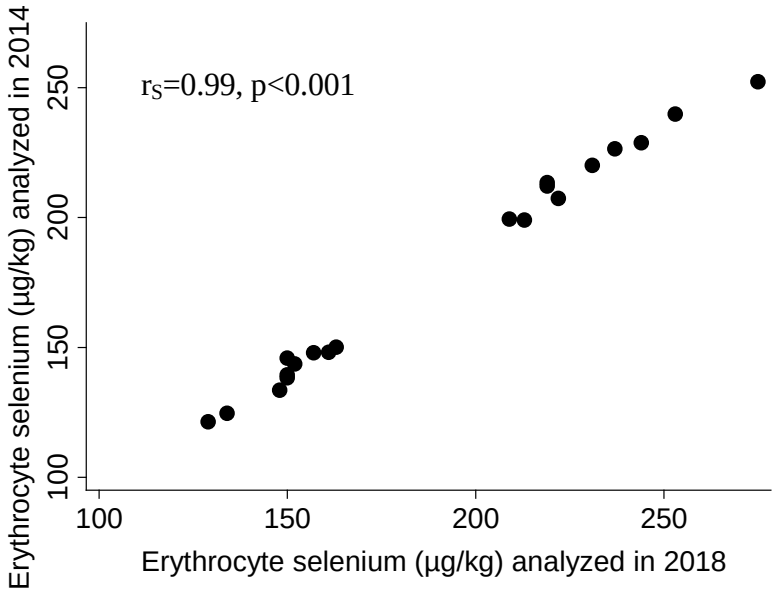

Figure 9. Erythrocyte selenium concentrations (µg/kg) in 20 samples reanalyzed after almost four years in -80°C.

4.5 OUTCOMES

Children were followed-up at 1.5, 5, and 10 years for developmental assessment (developmental cohort), as well as at 4.5 and 9 years for biomarker evaluation (immune cohort, mentioned under 4.3.1).

4.5.1 Cognitive abilities

At 1.5 years (**paper I**), child development (mental development and psychomotor development) was assessed using a revised version of the Bayley Scales of Infant Development (BSID-II; Saha et al. 2010). The tests were conducted by psychologists at the local health care facilities, and involved tasks such as problem solving and vision fixation. These procedures were videotaped and scored, and the scores from each test were converted into a total z-score. The test was modified and adapted to the Bangladeshi culture based on piloting in rural areas. The changes were limited to the minimum required and included a few pictures used to assess the mental development. In addition, children's language development was assessed using an inventory specially developed for Bangladesh (Hamadani et al. 2010a). This inventory was based on the principles of the MacArthur Communicative Development Inventory (Fenson et al. 2002), in which the score is constructed from children's recognition and usage of words and actions, according to the mother's report.

At 5 years (**paper III**), children's cognitive function was assessed using the third edition of the Wechsler Pre-school and Primary Scale of Intelligence (WPPSI) at the nearest health care facility (Wechsler 2002). WPPSI was culturally adapted and modified for use in Bangladeshi children (Hamadani et al. 2011). Seven subtests of WPPSI were used; information, vocabulary and comprehension were summed to form the verbal score, while block design, matrix reasoning and picture completion formed the performance score. Finally, processing speed as well as the verbal and performance score were summed to generate the children's full developmental score.

At 10 years (**paper III**), the Wechsler Intelligence Scale for Children, 4th edition (WISC-IV) was used. This was also culturally adapted to fit the present population with slight changes in the questions and translation to Bengali. The test generates four scales; verbal comprehension (based on vocabulary, information and comprehension), perceptual reasoning (based on block design, picture concept and matrix reasoning), working memory (based on digit span and arithmetic's), and processing speed (based on coding and symbol search). In addition, the full developmental score was calculated (sum of sub-scores), which represents the child's general intellectual ability. The raw scores of each test (both at 5 and 10 years) were used to exclude bias due to comparison with other culture norms.

4.6 ETHICAL CONSIDERATIONS

All studies have been ethically approved by the Research and Ethical Review Committees of icddr, b (Bangladesh), and the Regional Ethical Review Board of Karolinska Institutet, Stockholm, Sweden.

Written and oral consent was obtained from the mothers before enrollment into the MINIMat trial. In addition, parents or guardians gave their written consent prior to the children's participation at each follow-up. Women and children were informed about their option to refrain from the study at any time point without affecting their access to routine health services.

There are no risks with donating urine samples. For blood, there is a small risk for bleeding, bruising, or a local inflammation. However, sterile needles were used (eliminating the risk of spreading any contagious diseases), and blood was drawn by experienced staff to minimize discomfort. In order to lower the study burden for each child, the cohort was divided in two parts, one with focus on toxic mechanisms and one focusing on effects on child development.

All of the results concerning measured exposures and associations with the various outcomes have been reported to responsible PIs and other involved researchers at icddr,b. Because most of the analyses and evaluations were performed long after the follow-ups, it was not possible to report the results back to the families. However, in case of abnormal results, this was communicated to the responsible PI at icddr,b. The initially measured arsenic concentrations in the well water, usually one per family, were communicated by painting the pumps read [high arsenic, >50 µg/L (national cut-off)] or green (low arsenic, <50 µg/L). Also, in collaboration with BRAC, a non-governmental organization, the public was informed about

the health risks with arsenic in the drinking water in each village and recommendations were given for pregnant women and children, in particular, to collect drinking water from a green-painted well. Later, many families had deeper wells installed to decrease the arsenic concentrations in the drinking water, and we assessed the effectiveness of this mitigation effort by analyzing the arsenic concentrations in water and urine (Kippler et al. 2016b).

4.7 STATISTICAL ANALYSES

For a more detailed description of all statistical methods used, the reader is referred to each individual study (**paper I-IV**).

All statistical analyses were performed using Stata/IC (version12; StataCorp). Throughout **paper I-IV** we used complete subject analyses, i.e. participants with missing covariate data were excluded, assuming these data were missing at random. Bivariate associations between continuous variables were assessed using Spearman's correlation coefficient, while those between continuous and categorical variables were assessed using Kruskal-Wallis, Mann-Whitney *U*-test, chi-squared, or Fisher's exact test. Scatter plots with Lowess moving-average lines were used to visually examine all studied associations. For **paper II** and **IV**, we $\log_2$-transformed the concentrations of multiple elements due to skewness, which also simplified the visualization of any associations with these elements.

All associations evaluated in the present thesis were assessed using multivariable-adjusted regression analyses. Linear regression was applied when associations appeared linear, while linear spline regression with one or two knots was used to evaluate indicated non-linear relationships. For comparison of goodness of fit for linear *vs* spline models, we used the F-test, and chose the model with significantly higher R^2. For regression diagnostics, we used fitted *vs* residual plots and q-q plots. The results are presented as B-coefficients (non-standardized) and 95% confidence intervals (CI).

The regression models were adjusted for variables that are known to affect the outcomes, or that correlated with both selenium status and any of the outcomes in each respective study. We also applied backwards elimination starting with the variable with the highest p-value, and dropped variables if the R^2 was significantly improved after exclusion. For highly correlated variables (r_S>0.60), we included the variable that resulted in the highest R^2.

We performed various sensitivity analyses to assess potential mediation, and to determine the effect of extreme values on the overall results. In addition, we stratified on population groups (e.g. by gender or SES) to assess potential effect-modification, and included multiplicative interaction terms in the non-stratified models. Differences between estimates were tested using the Wald test. A p-value <0.05 was considered statistically significant, except for interaction terms, for which a p-value<0.10 was considered significant.

5 RESULTS AND DISCUSSION

In order to increase our understanding of the importance of selenium for early-life development, we initiated the studies described below, which are based on an ongoing mother-child cohort in rural Bangladesh, enabling prospective evaluation of selenium status and children's cognitive development. Given the lack of knowledge on the impact of adequate selenium status for early-life development, the most important findings of the present thesis are the positive associations between selenium status and children's cognitive abilities. The main results from each of the papers (**I-IV**) are presented below, as well as some unpublished data. For more details, please see each respective paper.

5.1 SELENIUM STATUS AND COGNITIVE FUNCTION

5.1.1 Maternal selenium status and children's cognition

In the first study (**paper I**), we evaluated the importance of prenatal selenium for the cognitive abilities in early childhood. We found a positive association between maternal erythrocyte selenium, measured in the third trimester (GW30; range 0.19-0.87 µg/g Hb; corresponding to 78-335 µg/L), and all measures of children's cognitive function, but psychomotor development and language comprehension in particular, assessed at 1.5 years (n=729; Figure 10). For mental development index and language expression, we found no significant associations, although the estimates were in general positive. The regression analyses were adjusted for age at testing, gender, gestational age at birth, HOME, weight-for-height z-score, birth weight, maternal age, family SES and maternal BMI. In two additional models, we further adjusted for other essential (iodine, manganese, and zinc) and toxic (arsenic, cadmium, and lead) elements.

In the stratified analysis, the association with psychomotor development appeared stronger for the girls; the estimate was 2.6 times higher than that for the boys. For language comprehension, on the other hand, the associations appeared slightly stronger among boys (1.7 times higher estimate). However, after now repeating this analyses and including an interaction term (child gender × erythrocyte selenium), we find no significant interaction for any of the outcomes ($p>0.25$ for all), implying that the estimates for girls and boys were not statistically different. This may suggest that the differences in estimates were random, although a larger sample size would have resulted in more narrow confidence intervals, which would have clarified this.

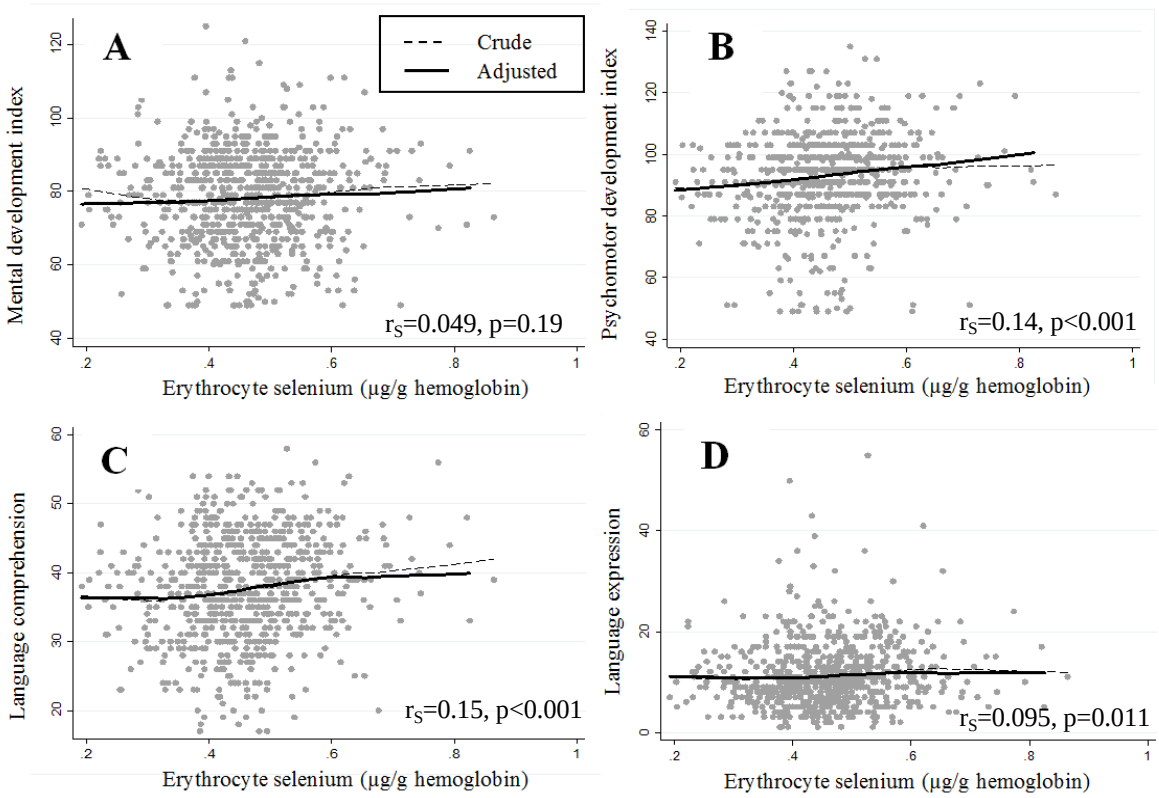

Figure 10. Correlations [crude lowess line (dashed) and adjusted lowess line (solid)] between erythrocyte selenium (µg/g Hb) at gestational week 30 and mental development index (A), psychomotor development index (B), language comprehension (C) and language expression (D). The adjusted lowess line is adjusted for age at testing, gender, gestational age at birth, HOME, weight-for-height z-score, birth weight, maternal age, family SES and maternal BMI.

For language comprehension, there was an indication of a non-linear relationship, why we used linear spline regression with two knots for the evaluation. We have also repeated the analyses using linear regression (no spline knots) and found that the predictive value of the model is actually marginally higher than that for the linear spline model (R^2=0.353 *vs.* R^2=0.351). This model also revealed that the association between maternal erythrocyte selenium and children's language comprehension remains significant (with the same effect estimates) regardless of adjustment for other essential elements (corresponding model 3; B=3.1, 95% CI: 0.16, 6.0, p=0.039) or toxic elements (corresponding model 4; B=3.5, 95% CI: 0.84, 6.1, p=0.010). We now also included an interaction term between maternal erythrocyte selenium and supplementation group, and additionally adjusted all analyses for supplementation group. We found no difference in the estimates (<7% difference between adjusted and non-adjusted models) for erythrocyte selenium at GW30 (further discussed under 5.2), and no interaction between selenium concentration and supplementation group.

At the follow-ups at 5 and 10 years, we found that the positive associations between maternal selenium status during pregnancy and children's cognitive abilities were still present (**paper III**). The associations were less precise at the 5 year follow-up compared to that at 10 years. It is well established that the older a child is at the time of cognitive testing, the better is the prediction of later life cognitive function. In general, stability improves after 6 years of age

(Berk 2013), which could explain why the associations between maternal selenium status during pregnancy and children's cognitive abilities assessed at 5 years were more imprecise than those assessed at 10 years. Throughout all follow-ups, we generally found stronger estimates for girls compared to boys. However, at 5 and 10 years, the differences were small and not significant. Consequently, the data indicates that adequate early-life selenium status is important for cognitive development in both girls and boys. However, in **paper IV** (discussed further in section 5.3), we found that the selenium retention appeared to be higher in girls than in boys, although we could not find any apparent reason for this. Differences in selenium metabolism could impact the health effects of selenium, and thus, potential gender difference should be assessed further in future studies.

We found no significant differences in selenium-related estimates for the outcomes at 10 years between children born preterm/term, that were stunted/normal height, or that had low/high SES ($p>0.18$ for all interaction terms for full score 10 years), implying that selenium is important independent of these factors.

At 1.5 years, the increase in psychomotor development and language comprehension comparing the 5^{th} to the 95^{th} percentile of maternal erythrocyte selenium corresponded to ~0.5 and 0.3 SD of the respective outcomes. At the follow-up at 5 years, the corresponding estimate for full developmental score was ~0.2 SD, and at 10 years ~0.3 SD. Taken together, the associations between prenatal selenium status and children's cognitive abilities appear robust over time and independent of other factors.

Since **paper I** was published (2015), other prospective studies have reported similar findings, but also contradictory results (Table 5). A large study performed in the U.S. found no association between maternal erythrocyte selenium at GW30 and child cognitive abilities at 7.7 years, however, the selenium concentrations were generally quite high (Oken et al. 2016). Indeed, the studies reporting a positive association between maternal/cord selenium concentrations and child cognition/neurodevelopment have all included rather low concentrations (Kippler et al. 2016a; Polanska et al. 2016; Snoj Tratnik et al. 2017; Varsi et al. 2017), while those reporting inverse associations have reported higher concentrations (Boucher et al. 2014; Oken et al. 2016), and those around sufficient concentrations have indicated non-linear associations (Amoros et al. 2018a; Amoros et al. 2018b). This would suggest that the association is in fact non-linear, although the turning point is yet to be confirmed.

Table 5. Summary of studies on prenatal selenium status and children's cognitive abilities/neurodevelopment.

Country	Age	Selenium assessment	n	Results		Comments	Reference
Bangladesh	1.5 years	Maternal erythrocytes: median 0.46 µg/g Hb	606-713	+ /	Positive associations between erythrocyte selenium (GW30), psychomotor development and language comprehension (significant), and mental development and language expression (non-significant) at 1.5 years.	Strongest association in girls. Indicated non-linear association for language comprehension.	**Paper I**
Bangladesh	5-10 years	Maternal erythrocytes: median 0.45 µg/g Hb	1260-1408	+	Positive associations between erythrocyte selenium (GW14) and cognitive outcomes both at 5 and 10 years.	Associations more clear for outcomes assessed at 10 years.	**Paper III**
Canada	6.5-11 months	Maternal plasma: mean 296.4 µg/L	94	/	No correlation between cord blood selenium and cognitive function.	Analyses not adjusted for any confounders. Extremely high selenium concentrations.	Boucher et al. 2014
China	3 days	Cord serum: mean 63.1 µg/L	927	+	Positive association between NBNA score and cord serum selenium up to 100 µg/L.	Potential inverse association above 100 µg/L (n=80; inverted U-shape).	Yang et al. 2013
Greece	4 years	Maternal urinary selenium: mean 23 µg/L	575	+	Positive associations with children's general cognitive score.	Borderline significance.	Kippler et al. 2016
Norway	6 months	Maternal serum: mean 72 µg/L	105	+ /	Positive association between maternal serum selenium at GW18 and children's ASQ total, problem solving, and fine motor scores.	No association with other sub-scores and not with serum Se measured at GW28 or GW36.	Varsi et al. 2017
Poland	1-2 years	Maternal serum 1st trimester: mean 48.3 µg/L	410	+	Positive associations between plasma selenium (first trimester) and children's psychomotor abilities within the first 2 years of life.	No measurements of maternal IQ, home environment, or other essential or toxic elements.	Polanska et al. 2016
Slovenia and Croatia	1.5 years	Cord blood serum: geometric mean 40.1 µg/kg	280	+ /	Borderline significant association with language composite score. Associations with other scores not reported.	Association with selenium not primary outcome: reported estimate from adjusted Hg-model. Potential influence of ApoE genotype.	Snoj Tratnik et al. 2017
Spain	1 year	Maternal serum around GW13: mean 79.7 µg/L	651 or 349	/ -	Non-linear model showed best fit. Inverse association with mental development indicated above 86 µg/L.	Suggested different shapes depending on *INMT* genotype.	Amoros et al. 2017
Spain	5 years	Maternal serum around GW13 : mean 79.9 µg/L	490	+ / -	Non-linear model showed best fit with verbal and global memory scales, break point at 84 µg/L.	Effect modification by breast feeding suggested.	Amoros et al. 2018
Sweden	5-17 years	Cord serum: mean 48 µg/L; maternal serum: mean 72 µg/L	180 cases, 191 controls	-	Increased odds of having ADHD with high selenium concentrations.	Authors have no explanation for their results and suggest chance finding.	Ode et al. 2015
U.S.	7.7 years	Maternal erythrocytes: mean 206 µg/L	872	/	No association between maternal erythrocyte selenium at GW30 and child verbal or non-verbal intelligence, visual motor function, or visual memory.	High selenium concentrations.	Oken et al. 2016

+ positive association, - inverse association, / no association. ADHD, Attention Deficit Hyperactivity Disorder; ASQ, Attachment Style Questionnaire; GW, gestational week; Hb, hemoglobin; INMT, indolethylamine N-methyltransferase; IQ, Intelligence quotient; NBNA, Neonatal Behavioral Assessment Scale.

5.1.2 Children's selenium status and cognition

5.1.2.1 Positive associations

In **paper III** we found that also the children's selenium status during childhood was associated with their cognitive abilities (Figure 11). Because of the lack of blood samples from the children in the developmental cohort, we assessed the status based on concentrations in urine and hair. The suitability of these biomarkers of selenium status is discussed in the following sections (5.3.1 and 5.3.2). The strength of the associations were similar at 5 and 10 years when using urinary selenium, but were stronger when using hair selenium, the more long-term marker (10 years). In addition to the cross-sectional analyses, we also found an association between urinary selenium at 5 years and the full developmental score at 10 years.

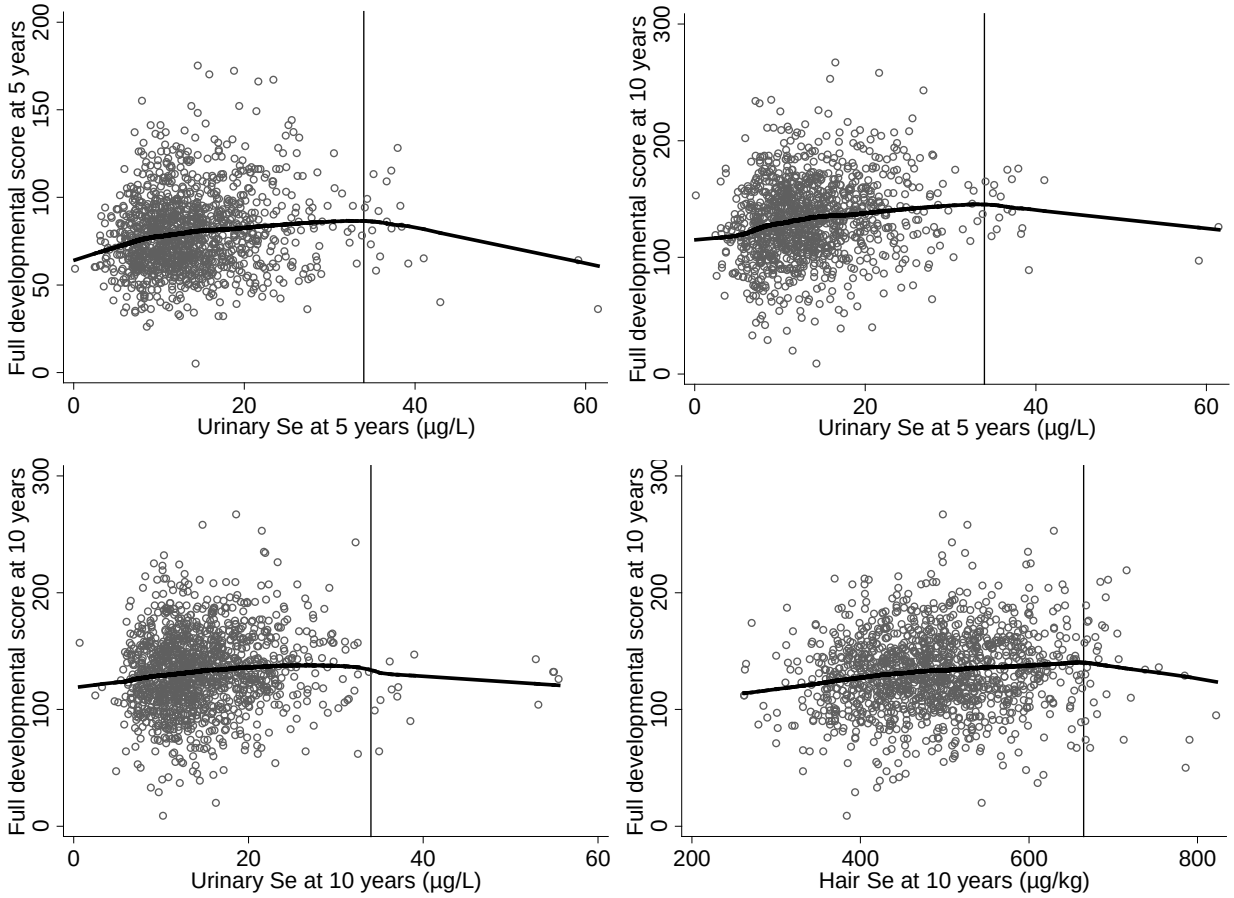

Figure 11. Scatter plots with smoothed lowess lines for childhood selenium (Se) and full developmental score (raw score) at 5 and 10 years. The vertical lines represent the turning point used for the knot in the linear spline regression analyses (34 µg/L for urinary selenium at 5 and 10 years and 665 µg/kg for hair selenium at 10 years).

For an increase from the 5th to the 95th percentile in hair selenium, the corresponding increase in full developmental score at 10 years was about 0.2 SD, i.e. similar to the effect estimate for maternal selenium status in pregnancy. We now also included selenium in both hair (10 years) and erythrocytes (GW14) in the analyses, and found that the associations with full developmental score persisted for both time point with similar effect estimates to those from

the separate models. Indeed, this supports the interpretation that adequate selenium status is of importance for brain development throughout childhood. Similar to the analyses including prenatal selenium, we found no evidence of effect modification for the association with hair selenium by gestational age at birth, SES, growth (HAZ), or gender ($p>0.31$ for all interaction terms for full score 10 years). Given the wide variations in these variables in the present cohort, the results indicate that the associations between selenium status and child cognitive development may be applicable also to other populations (further discussed under section 5.4.4).

For comparison with the present results, we have reviewed the few available studies that have assessed the impact of childhood selenium status on cognitive abilities, but also on motor function, with varying results (Table 6). Unfortunately, several of the studies included in Table 6 did not have selenium as the primary exposure, and have not assessed the relationship thoroughly, but merely through Spearman/Pearson correlations with the outcomes (Bumoko et al. 2015; Gashu et al. 2016; Gassio et al. 2008; Sun et al. 2015). Also, the studies are generally small. A study from Brazil found a positive association between plasma selenium and motor function, even though the mean plasma concentration was as high as 163 µg/L (Lemire et al. 2011). However, this study included participants between 15-87 years of age, and the requirement for selenium might be higher for older individuals, which could be driving the positive association. Another study from Bangladesh presented a positive association between selenium status and children's motor function at 9.6 years of age in one of the sub-tests (Parvez et al. 2011), and the concentrations were similar (mean 105 µg/L in whole blood) to those estimated in the present population [119 µg/L (**paper IV**), assuming a hematocrit (%) of 3xHb (g/dL; Dosoo et al. 2014)].

The effect estimates from **paper I** and **III** may seem small, and on an individual level such changes in cognitive abilities (corresponding to 3-5 IQ-points for a change from the 5^{th} to the 95^{th} percentile) will not be of high clinical relevance. However, on a population level, even small changes in cognitive abilities are of importance as this may impact the frequency of children falling below the limit for intellectual disability. Importantly, it has been estimated that 200 million children under five years in the world do not reach their full cognitive developmental potential due to preventable factors such as nutritional deficiencies (Grantham-McGregor et al. 2007). Indeed, the results from **paper I** and **III** add support for interventions promoting better nutrition and education. As stated by others, "*Interventions to promote early child development are cost-effective investments to ensure that children are prepared for educational and economic opportunities* […]" (Engle et al. 2007).

5.1.2.2 Indicated toxicity

At both 5 and 10 years, there were indications of non-linear associations between childhood selenium status and cognitive abilities (Figure 11). However, it should be noted that there were few children with concentrations above the indicated turning points (n=18-34). Even so, the non-linear associations seemed to have the same turning point at both 5 and 10 years (around 34 µg/L in urine), and was also present when using hair selenium at 10 years (more

long-term marker; turning point at 665 µg/kg). Using the equation for predicting hair concentrations from plasma concentrations found in **paper IV** (discussed under section 5.3.1), a hair concentration of 665 µg/kg would correspond to 174 µg/L in plasma. However, this is extrapolated beyond the range of plasma selenium for the children included in **paper IV**, and it should therefore be interpreted with caution. For the association between urinary selenium at 5 years and cognitive abilities at 10 years, there was also an indicated turning point at 34 µg/L, however, the spline model and linear model were not significantly different in goodness of fit.

When comparing these finding to others (Table 6), we found a Canadian study reporting an inverse association between children's blood selenium and the latency of visual evoked potentials. However, the children had very high selenium concentrations (mean whole blood concentration: 331 µg/L; Saint-Amour et al. 2006), and the study included only 78 children. In addition, the inverse associations in **paper III** included only 18, 20, or 34 children (above the turning points), why this need to be more thoroughly assessed in future studies.

Taken together, there are indications that the relationship between selenium status, both prenatal (as discussed in section 5.1.1) and during early childhood, and neurodevelopment could be non-linear. Indeed, it is known that selenium may act as a pro-oxidant at high intake (Lee and Jeong 2012), which may be the reason for the inverse associations. Indeed, recommendations of selenium supplementation, especially in the form of pills, should always be accompanied by information about the risk of toxic effects at over-dosing. More is not always better.

Table 6. Summary of studies on childhood selenium status and cognitive/motor/neurological abilities.

Country	Age	Selenium assessment	n	Results		Comments	Reference
Bangladesh	5 and 10 years	Urine: mean 14 and 15 µg/L at 5 and 10 years Hair: mean 487 µg/kg at 10 years	1167-1330	+ / -	Positive association with cognitive abilities at both 5 (non-significant) and 10 years (significant).	Indicated non-linear associations at both ages, turning point at 34 µg/L for urine and 665 µg/kg for hair.	**Paper III**
Bangladesh	9.6 years	Whole blood: mean 104.9 µg/L	299	+ /	Positive associations with motor function. No significant association for remaining 4 outcomes.	Population highly exposed to arsenic.	Parvez et al. 2011
Brazil	15-87 years	Plasma: mean 163 µg/L; Hair: mean 840 µg/kg; Urine: mean 57 µg/g cr; Whole blood: mean 288 µg/L	448	+	Positive associations between plasma selenium and motor functions.	Associations stronger when adjusting for blood mercury. Only significant associations in stratum with high mercury.	Lemire et al. 2011
Canada	4.8-6.2 years	Whole blood: mean 401.6 µg/L	110	/	No association between selenium and motor function.	Very high selenium concentrations.	Despres et al. 2005
Canada	4.8-6.1 years	Whole blood: mean 331 µg/L	78	-	Positive association between blood selenium and latency.	Very high selenium concentrations. Small sample size.	Saint-Amour et al. 2006
China	8-12 years	Whole blood: mean 94.77 µg/L	446	/	No correlation between selenium and IQ.	Analyses not adjusted.	Sun et al. 2015
Ethiopia	54-60 months	Serum: median 61.4 µg/L	541	+	Cognitive deficits more common in selenium deficient children.	Unclear whether analyses is adjusted for potential confounders.	Gashu et al. 2016
Democratic Republic of Kongo	8.5 years	Plasma: mean 30 µg/L	123 cases 87 controls	+ /	Positive association between selenium and motor function in children with konzo.	Spearman correlation used to assess association. No association with cognition.	Bumoko et al. 2015
Spain	9.7 years	Plasma: mean 49 µg/L in PKU patients and 71 µg/l in controls	36 cases, 29 controls	+ /	Positive association between plasma selenium and performance in PKU patients (attention, impulsiveness, response time, etc).	No correlation between selenium and general intelligence in PKU patients.	Gassio et al. 2008

+ positive association, - inverse association, / no association Abbreviations: cr, creatinine; IQ, Intelligence quotient; PKU, phenylketonuria

5.2 MATENAL SELENIUM STATUS

The mean erythrocyte selenium concentration at GW14 was 152 µg/kg (corresponding to 0.45 µg/g Hb; **paper III**). Even though erythrocyte selenium is considered a valid, long-term marker of selenium status, there are no reference values established. For some of the women (n=98), we also had plasma selenium in early pregnancy, and the average concentration was 60 µg/L (Li et al. 2008). This has been designated as low (Van Dael and Deelstra 1993), especially in relation to maximizing selenoprotein P, which occurs at higher concentrations (80-125 µg/L; Hurst et al. 2010). Even in relation to optimization of GPx3, which occurs at 40-60 µg/L (Burk and Levander 2006), a large part of the women appeared to have low selenium status. As mentioned in section 2.1.5, this reasoning has lately been challenged, and the recommendations might be lowered in the future. To conclude, based on current cut-offs, a poor selenium status appeared prevalent among the pregnant women participating in MINIMat, as 60% of them had concentrations <60 µg/L, and 95% of them had concentrations <80 µg/L. However, it should be noted that such concentrations are also fairly common in many other countries, e.g. in large parts of Europe and south-east Asia (Figure 12).

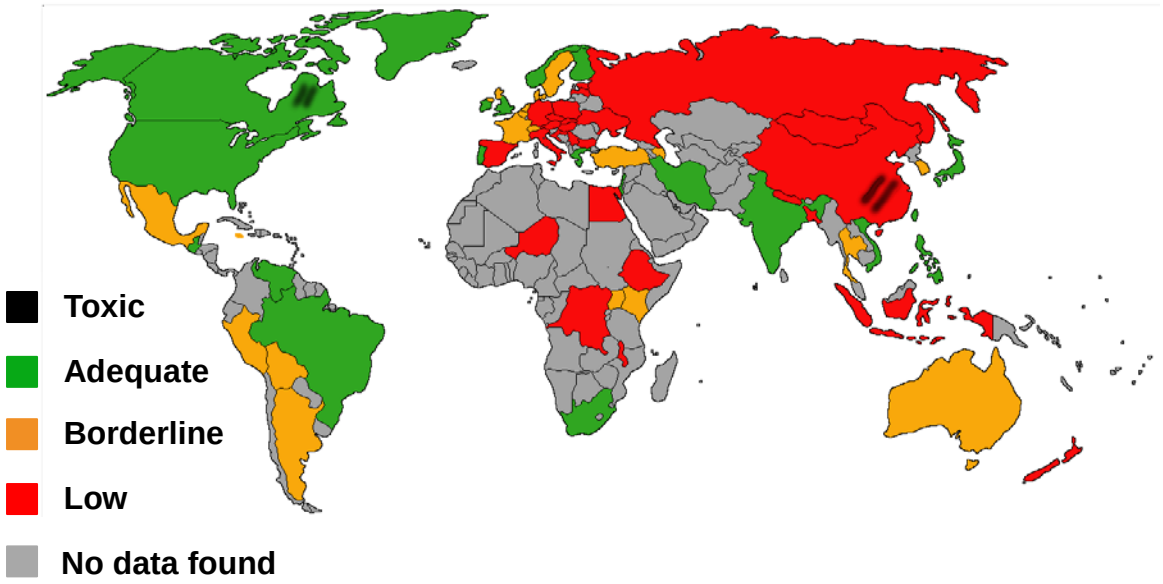

Figure 12. Selenium status across the world based on mean plasma concentrations among healthy populations. The cut-offs are suggested based on maximization of selenoprotein P; low (<80 µg/L), borderline (80-100 µg/L), adequate (>100 µg/L), and toxic (intake over 850 µg/day). Data from: Abdulah et al. 2013; Chen et al. 2006; Choi et al. 2016; Combs 2015; Gashu et al. 2016; Gibson et al. 2011; Golubkina and Alfthan 2002; Hagmar et al. 1998; Haldimann et al. 1996; Imai et al. 1995; Kishosha et al. 2011; Kornhauser et al. 2008; Kuehnelt et al. 2015; Lander et al. 2008; Lemire et al. 2011; Li et al. 2008; Maduray et al. 2017; Pritchett et al. 2017; Schulze et al. 2014; Stoffaneller and Morse 2015; Van Nhien et al. 2006; FAO/WHO 2002; Saint-Amour et al. 2006.

The Spearman correlation between the concentrations at GW14 and GW30 (overlap between **paper I and III**, n=444) was 0.44 ($p<0.001$), and the concentrations were lower in the third trimester (mean 144 µg/kg) compared to early second trimester (mean 158 µg/kg; $p<0.001$). Indeed, blood selenium has previously been shown to decrease during pregnancy, probably partly due to the demand of the fetus (Burk and Levander 2006). Surprisingly, we found no difference in erythrocyte selenium at GW30 by the different supplementation groups (**paper I**). The reason for this is unknown, but could be related to the absorption being lower for selenite than for organic forms such as selenomethionine (Roman et al. 2014). In addition, it is possible that the absorption is also affected by the combination of the 14 other micronutrients present in the pill.

Using the plasma concentrations in early pregnancy in the sub-sample of women, we could also assess the relationship between erythrocyte and plasma concentrations. These were strongly correlated; $r_S=0.69$, $p<0.001$ (**paper I**), and the best fitted equation (1) for the prediction of erythrocyte selenium from plasma selenium was the following:

Erythrocyte selenium (µg/kg) = 2.47 x Plasma selenium (µg/L) + 25.5 (1)

This implies that a plasma concentration of 60 µg/L (commonly used as cut-off for deficiency) would correspond to a concentration of 174 µg/kg in erythrocytes. Comparing this concentration to those of all women included in **paper I** (n=750, GW30) **and III** (n=1408, GW14) suggests that 92% and 75% were selenium deficient at the time of sampling, respectively.

Unexpectedly, the urinary selenium concentrations appeared to increase across pregnancy, with the largest increase in early pregnancy (Figure 13). Possibly, this can be explained by the increase in glomerular filtration rate during pregnancy (Dunlop 1981). Also, we recently found that the relative concentration of TMSe decreased during pregnancy (Skröder et al. 2018), implying that the production of other selenium metabolites (e.g. selenosugars) increased, which may be related to an upregulation of the one-carbon metabolism (Gardner et al. 2011). Such an upregulation would also increase the production of TMSe, and the concentration of this metabolite did in fact increase across pregnancy, although the relative amount decreased. Thus, this is an additional potential mechanism for the increased urinary selenium concentrations across pregnancy.

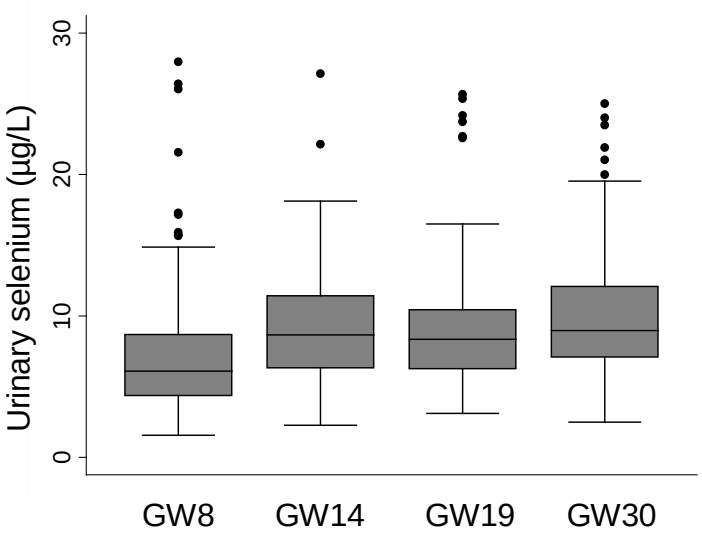

Figure 13. Urinary selenium concentrations across pregnancy among women who donated urine- and blood samples at all follow-ups during pregnancy (n=155).

The correlation between the selenium concentrations in erythrocytes and urine in the sample of women with measurements at several time points throughout pregnancy (n=155) was very weak: r_S=0.13 (p=0.11) at GW14, and r_S=-0.086 (p=0.50) at GW30. The likely reasons for this poor correlation include the increased demand of the fetus, the increase in GFR, as well as the fact that urinary selenium is a short-term marker of selenium intake (days), in contrast to erythrocyte selenium (months; Thomson 2004). In particular the latter is supported by the fact that the correlation was stronger when comparing the urinary concentrations to the plasma concentrations at GW14 (r_S=0.31, p=0.045) in the sub-sample of women described above (n=98), as plasma concentrations reflect intake over the past week/weeks (Hawkes et al. 2008). However, the correlation was still rather weak. This may be a result of the plasma volume expansion, which starts already between GW6-GW10 (Faupel-Badger et al. 2007).

Nevertheless, given the indicated low selenium status in the pregnant women, and the implied importance of selenium for fetal brain development, it appears disadvantageous to increase the loss of selenium during pregnancy. The potential mechanisms and health consequences of this loss need further research.

5.3 CHILDREN'S SELENIUM STATUS

5.3.1 Biomarkers

The urinary selenium concentrations in the children both at 5 (mean 14 µg/L) and 10 years (mean 15 µg/L; **paper III**) were quite low, but still higher compared to the mothers during pregnancy (mean 7.2 µg/L). Again, there are no cut-offs established for urinary selenium, but 26 µg/L has been suggested to indicate selenium sufficiency as this has been found in selenium adequate areas (Högberg and Alexander 2007). At both 5 and 10 years, 95% of the children had concentrations below this value, which would indicate low selenium intake.

Thus, it was surprising to find that only 3% of the children with available plasma samples at 9 years (n=223, **paper IV**) had concentrations below 60 µg/L (41% below 80 µg/L). A further discussion on factors influencing the biomarker kinetics in the children, including urinary excretion, is provided in the following section (5.3.2).

The erythrocyte concentrations at 4.5 and 9 years (**paper II and IV**) were also higher (mean 176 µg/L at 4.5 years and 189 µg/L at 9 years) than for the mothers even in early pregnancy, which does indicate a better selenium status in the children compared to their mothers. Part of the difference in selenium status between mothers and children is probably due to the pregnancy-related influences described above. Indeed, plasma selenium has been found to be approximately 20% lower among pregnant women, compared to non-pregnant (Neve 1991). However, it is also possible that the intake of selenium has increased over time. In support, the prevalence of stunting (HAZ<-2) was lower at 10 years (~28%) than at 5 years (~33%; **paper III**) and at 1.5 years (~49%), indicating better general nutritional status.

When assessing whether hair could be used as a marker of internal dose of multiple elements (including selenium) for the present children (**paper II**), we found an overall correlation of r_S=0.38 (p<0.001) between the selenium concentrations in hair and erythrocytes. In addition, the correlation was stronger for samples representing the time of blood collection (the 7^{th}-8^{th} cm of hair; r_S=0.54, p=0.026). The mean selenium concentration in the children's hair at 9-10 years was 519 µg/kg (**paper II**, n=207) and 487 µg/kg (**paper III**, n=1330). The concentrations did not vary much within individuals (Figure 14; intraclass correlation coefficient=0.80, p<0.001), indicating low variation in selenium intake over time.

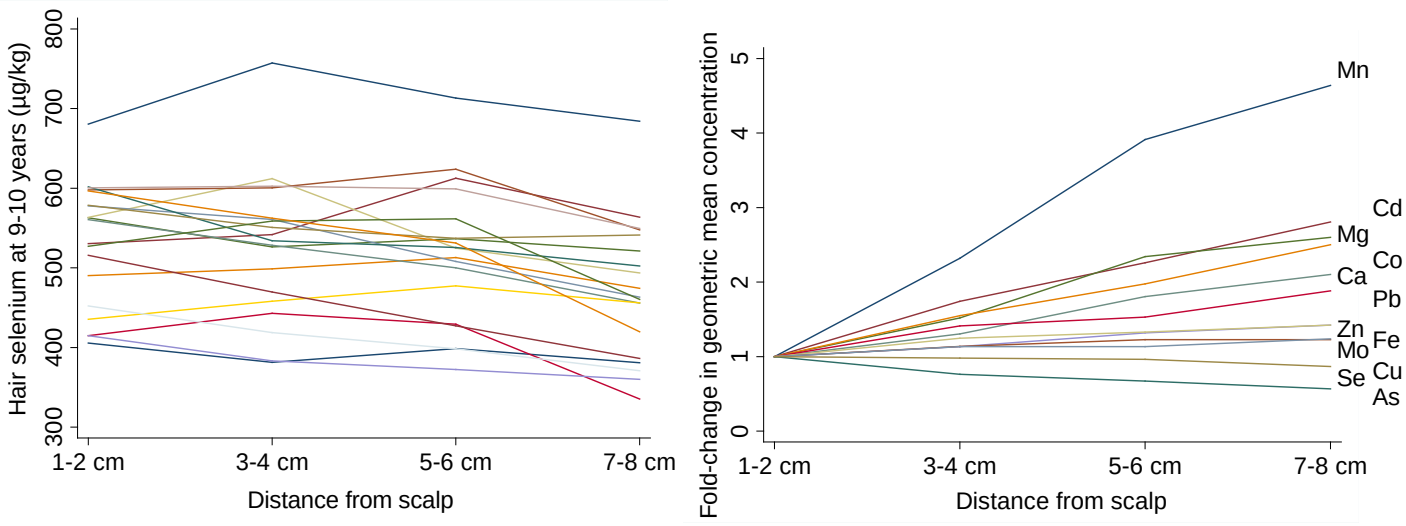

Figure 14. Element concentrations in hair from 19 girls analyzed in four sections of 2 cm each, counting from the head outwards. The left figure shows the concentrations of selenium along the hair, and the right figure the fold-change in geometric mean concentrations of all elements analyzed.

Hair selenium (as well as hair arsenic) did not seem to be markedly affected by external contamination. In contrast, the geometric mean concentration of manganese was almost 5-fold higher in the sample 7-8 cm from the head, compared to the first 1-2 cm (Figure 14).

One child in particular had very high manganese concentrations in all hair samples, and also in water used for both drinking and washing, but the concentration in erythrocytes was normal. We found strong associations between the arsenic concentrations in all media, but for the other elements, we found no/weak associations between hair and blood/urine, indicating that hair concentrations of these elements are not reflective of the internal dose. An illustration of the conclusions from **paper II** is shown in Figure 15.

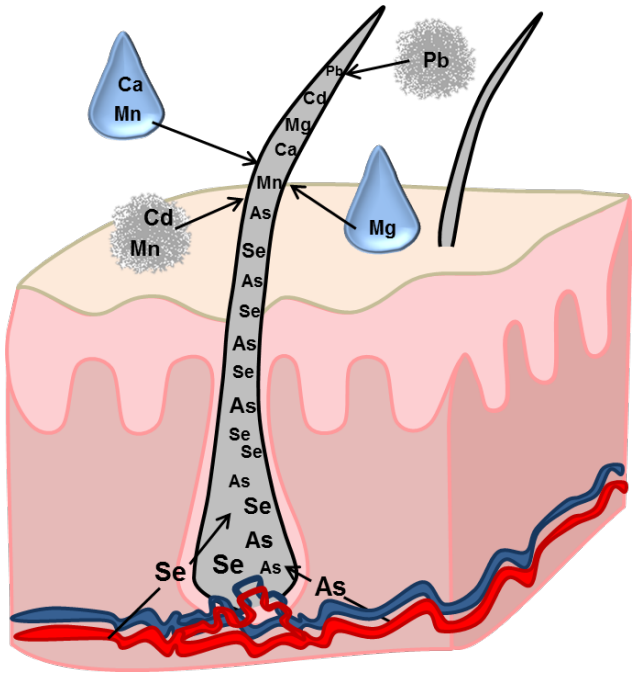

Figure 15. Summary of the findings from **paper II**. Selenium and arsenic in hair was found to represent the internal dose (concentrations in blood), while other elements in hair seemed to originate from external sources (dust or water).

The literature including measurements of hair selenium is large, but there is no consistency regarding the relationship with e.g. plasma selenium. Studies have reported very different concentrations of hair selenium even at similar plasma selenium concentrations (Figure 16), which means that other factors may influence the selenium concentrations in hair (section 5.3.2). In addition, a fairly recent JAMA-article showed that commercial laboratories (n=6) in the U.S. varied two orders of magnitude in their measurement of selenium in hair samples from the same person (Seidel et al. 2001), indicating influence from e.g. washing procedure, sample preparation and analytical method and performance.

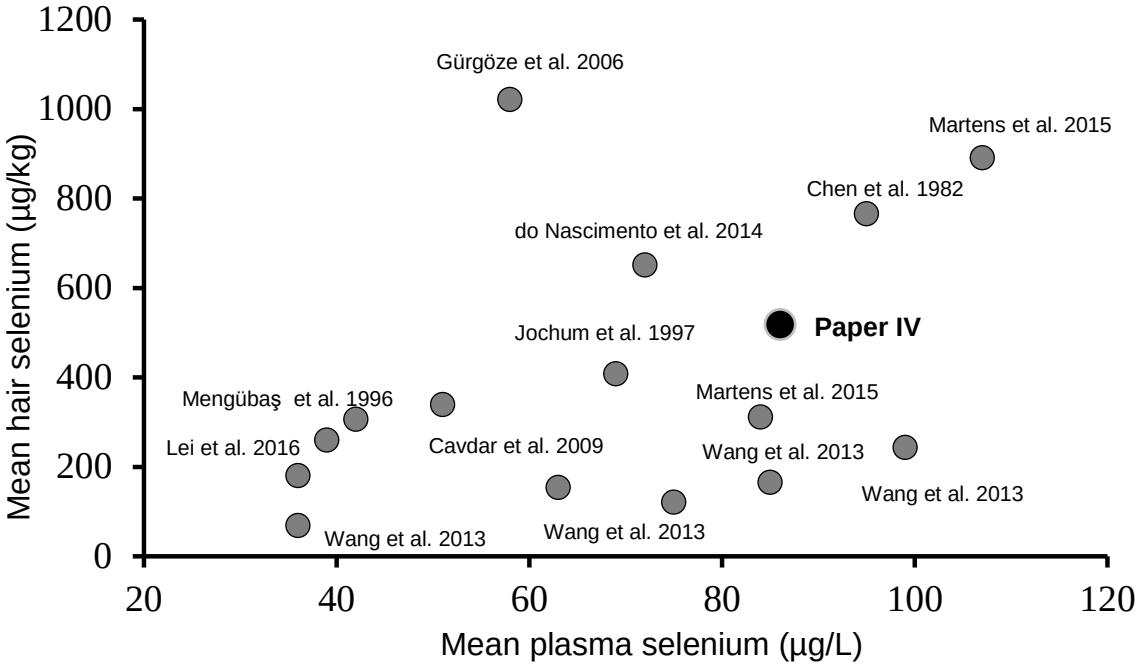

Figure 16. Mean concentrations of selenium in hair and plasma in studies including healthy children with both biomarkers analyzed.

For this reason, there are no established cut-offs for determining selenium deficiency, sufficiency, or toxicity, using concentrations in hair. The equation from the regression analysis in **paper IV** for hair and plasma selenium was as follows:

$$Hair\ selenium\ (\mu g/kg) = 1.8 \times plasma\ selenium\ (\mu g/L) + 351 \qquad (2)$$

This implies that for the present children, a plasma concentration of 60 µg/L would correspond to a selenium concentration of 459 µg/kg in hair.

5.3.2 Biomarker kinetics

Because of the observed uncertainties and discrepancies among the different biomarkers of selenium status used, we evaluated if any of all the available covariates could influence the selenium biomarker kinetics in the children (**paper IV**). Interestingly, we found that malnourished children seemed to retain more selenium (less selenium excreted in urine and more remaining in plasma), which is in line with the fact that selenium status is regulated through the urinary excretion (Robinson et al. 1985). Indeed, this is a likely explanation for the observation that only 3% of the children had low plasma selenium concentrations (<60 µg/L), while 95% had low urinary concentrations, as discussed above. It has been speculated that selenium becomes available for excretion first when selenoprotein production approaches saturation (Burk and Hill, 2015).

There is an important hierarchy of selenoproteins, both among the proteins and among tissues. At low selenium intake, the secretion of selenoprotein P from the liver to the plasma increases for maintenance of selenium transport to the most important tissues (Burk and Hill

2015), which may explain part of the higher P-Se in the malnourished children. In addition, a recent study on polymorphisms in genes related to selenoproteins indicated that humans living under selenium deficient conditions may adapt to such conditions (White et al. 2015). In fact, polymorphisms in such genes have been associated with both plasma and urinary selenium concentrations (Combs et al. 2011).

Also, we showed, for the first time, that polymorphisms in *INMT* predicted concentrations in both urine and hair (but not the blood fractions), with higher concentrations among TMSe producers (*INMT* genotype AA or AG). This is in line with our findings among the pregnant women, for which we found no impact of *INMT* genotype on erythrocyte selenium concentrations (Kuehnelt et al. 2015). Still, potential health implications from being a TMSe producer and thereby excreting more selenium are currently unknown and should be studied further, especially since the frequency of producers was much higher in Bangladesh (33%) compared to Argentina (<5%) even though the selenium status was higher in Argentina (Kuehnelt et al. 2015).

Other new findings were that both arsenic and cadmium exposure were predictors of selenium kinetics. For arsenic, higher exposure was associated with more selenium in the erythrocytes compared to other compartments (Figure 17).

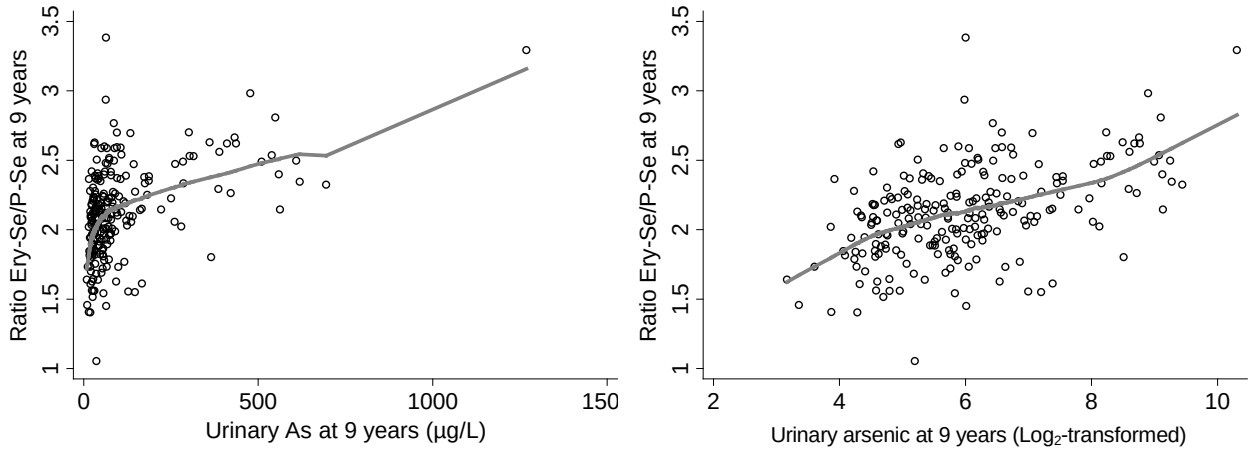

Figure 17. Scatter plots with smoothed lowess lines for the ratio of selenium in erythrocytes (Ery-Se) and plasma (P-Se) and urinary arsenic at 9 years of age (n=223).

There are multiple mechanisms involved in arsenic-selenium interactions. As arsenic is a pro-oxidant, the shift of selenium from plasma to erythrocytes with increasing arsenic exposure could be due to higher demand of GPx1, an antioxidative enzyme active in red blood cells. However, this needs to be further studied. The arsenic-selenium complex $[(GS)_2AsSe]^-$ that has been shown to form in erythrocyte lysate from rabbits (Manley et al. 2006) is another possible explanation for this association. Still, as mentioned under section 2.4.1, this has never been identified in humans, and the toxicological importance of such a complex would need to be assessed, given that the main excretory route for both arsenic and selenium is *via* urine.

Unexpectedly, cadmium was one of the strongest predictors of the selenium biomarkers. In contrast to arsenic, cadmium exposure (cumulative exposure assessed as urinary cadmium) was inversely associated with selenium in erythrocytes and positively with selenium in urine. As cadmium, another potent pro-oxidant, accumulates in the kidney and increases oxidative stress in this organ (Matovic et al. 2015), it is possible that the demand of selenium in the kidney increases with higher cadmium exposure in order to increase the expression of GPx3, which is produced in the kidneys (Avissar et al. 1994). A study on mice recently showed that selenium, in the form of selenoprotein P and small selenium-containing proteins, is filtrated through the glomerulus and reabsorbed in the proximal convoluted tubule through megalin-mediated endocytosis, and then used for production of GPx3 (Kurokawa et al. 2014). It has also been shown *in vitro* that cadmium may decrease the expression of megalin (Gena et al. 2010). Thus, it is possible that the positive association between urinary cadmium and selenium is explained by decreased reabsorption of both elements in the proximal convoluted tubule.

A large fraction of the variation in the biomarker selenium concentrations was still unexplained by the statistical models, most of which is likely explained by total selenium intake as well as sources and forms of selenium.

The findings of the impact on biomarker kinetics by arsenic and cadmium, the exposure of which is frequently elevated in the study area from contaminated drinking water (arsenic) and high rice consumption [both arsenic and cadmium (Kippler et al. 2010; Kippler et al. 2016b)], may indicate that selenium status is overestimated based on concentrations in erythrocytes or underestimated based on concentrations in urine. Also, the toxic exposures may increase the selenium requirement, for both mothers and children.

5.4 POTENTIAL MECHANISMS OF SELENIUM IN CHILD DEVELOPMENT

5.4.1 Thyroid function

The importance of selenium for the function of the thyroid was first discussed when a condition known as myxedematous endemic cretinism (resulting in mental retardation, short stature, goiter, and hypothyroidism) was described in the Democratic republic of Congo. This condition was characterized by selenium deficiency (Goyens et al. 1987), and since this finding, the interest for the importance of selenium for this organ has increased. In addition to the brain, selenium levels are conserved in the thyroid during periods of low intake. In fact, the thyroid is one of the organs with the highest amount of selenium per g tissue, together with the kidney (Duntas and Benvenga 2015).

The thyroid hormones are required already during the first trimester. However, the fetal thyroid function is not developed until gestational week 14-16 (de Escobar et al. 2004). Thus, the fetus is dependent on maternal thyroid hormones during the first trimester. After the fetal thyroid has started to produce these hormones, the fetus is instead dependent on maternal iodine and selenium for proper function. Still, the transfer of maternal T3 to the fetus is very

low (Velasco et al. 2018), and thus, the fetus is dependent on proper deiodinase function (and thereby selenium) also in early pregnancy, for the conversion of transferred T4.

Patients and animals with mutations in the *SBP2* gene, which encodes a factor essential for selenoprotein formation, are reported to have altered thyroid metabolism, reduced serum selenium and T3 levels, as well as deficits in motor coordination and mental ability (Pitts et al. 2014; Seeher et al. 2014). Also, selenium supplementation has been shown to be effective in treatment of several thyroid diseases (Ventura et al. 2017), although not in all studies (Mao et al. 2016).

The activity of the deiodinases has been found to saturate at plasma concentrations of 65 µg/L (Thomson 2004). In the small sub-sample of women with plasma selenium analyzed (n=98), 70% had concentrations below this level, suggesting that a large fraction of the pregnant women may have had an impaired thyroid function during pregnancy. However, the associations between maternal selenium status during pregnancy and children's cognitive development appeared linear across the whole range of (erythrocyte) selenium concentrations, indicating either that the deiodinases saturate at higher concentrations in the present population, or that other mechanisms are also involved.

In addition to selenium, adequate iodine is also essential for proper thyroid function, since this element is a component of T3 and T4. The two elements appear to collaborate in order to protect the levels of T3. In case of low iodine intake, iodine trapping increases, the synthesis of T3 is prioritized, and the conversion from T4 increases (Bougma et al. 2013). Therefore, the level of T3 is often not affected in many organs following low iodine intake. However, the brain seems to be more sensitive to low iodine status, and its levels of T3 falls below the normal even at mild iodine deficiency (Bougma et al. 2013). Thus, the conversion from T4 might be extra important in this organ. We did not yet assess the combined impact of selenium and iodine status on cognitive abilities in the present population. Still, we adjusted the analyses in **paper I** for maternal urinary iodine measured in GW8 to eliminate potential confounding since both elements are present in fish. This adjustment did not markedly influence the estimate for selenium. In **paper III**, we did not include the iodine adjusted models since the number of women and children with urinary iodine measurements were somewhat fewer than the sample included. However, adjusting any of the models (prenatal, 5-years cross-sectional, or 10-years cross-sectional) for urinary iodine at each respective time point did not have any major impact on the estimates for selenium (<1% difference between adjusted and unadjusted estimates). Importantly, it has been reported that when selenium and iodine deficiency are both present, correction of only the selenium deficiency has been associated with increased conversion of T4 to T3, which cannot be compensated by an increased T4 production due to the low iodine availability (Chanoine 2003). Therefore, it has been recommended that when intake of both elements is deficient, the iodine should be corrected prior to the selenium.

Unfortunately, we did not have measurements of thyroid hormones for the pregnant women. At 9 years, we did measure TSH in plasma available for 316 of the children in **paper IV** with hair selenium available. There was an indicated inverse association between hair selenium and TSH (Figure 18; implying better thyroid status at higher selenium concentrations), but unfortunately, it was not possible to analyze other thyroid hormones due to interactions with the Na-heparine from the tubes, why we could not assess this hypothesis further. Still, other studies on children's thyroid hormones and selenium status have suggested that suboptimal selenium intake has only a modest effect on thyroid function (Chanoine 2003).

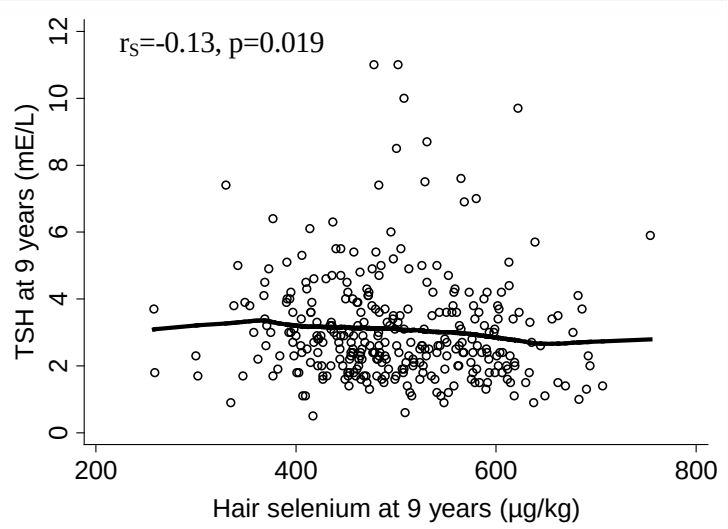

Figure 18. Scatter plot with smoothed lowess line for thyroid-stimulating hormone (TSH) and hair selenium at 9 years of age.

5.4.2 Antioxidative protection

Due to the high oxygen consumption of the brain, the production of ROS is particularly high in this organ (Roman et al. 2014). At imbalance with ROS-scavenging antioxidants, this can lead to oxidative stress, which has been associated with several neurodegenerative disorders (Roman et al. 2014). The developing brain is extremely susceptible to free-radical damage (Miller et al. 2012). Selenium, in the form of selenocysteine, is part of GPxs, which catalyze the breakdown of hydrogen peroxide. In addition, selenocysteine is also included in TrxRs, which are involved in the control of cellular proliferation and apoptosis through controlling thioredoxin activity and redox state (Fairweather-Tait et al. 2011). Besides GPxs and TrxRs, selenoprotein W, another selenoprotein with proposed antioxidative properties, is highly expressed in the developing as well as the adult brain, and the expression is maintained under dietary selenium deficiency in sheep and rats (Raman et al. 2013). The antioxidative actions of these proteins could be a mechanism for the observed, positive association between early-life selenium status and cognitive development. The positive associations were present for most of the assessed outcomes in **paper I** and **III,** indicating a general protective effect, i.e. that selenium is important for several areas of the brain. This is in line with the experimental studies described in section 2.3.1, in which changes in expression of various selenoproteins

(e.g. GPxs) affected both motor- and various cognitive functions. In addition, selenoprotein W was highly expressed in >90% of the brain regions of mice, including cortex, hippocampus, and cerebellum, further supporting a general effect.

In populations exposed to elevated levels of strong pro-oxidants such as arsenic and cadmium (Cuypers et al. 2010; Engstrom et al. 2010; Kippler et al. 2012a; Zwolak and Zaporowska 2012), which is the case in the present studies, a counteraction by selenoproteins may be a potential mechanism for the positive associations between selenium status and cognitive abilities. However, in both **paper I** and **III**, adjusting for exposure to toxic elements (arsenic, cadmium, lead, mercury, and manganese) had marginal impact on the estimates, implying that the positive association between selenium and children's cognitive abilities was independent of exposure to the toxic elements assessed. Still, many of the children in the present population are exposed to arsenic to a higher extent than most other populations (the range for the sum of iAs and its metabolites in urine samples from **paper IV** was 9-1268 µg/L). Also, because of the rice based diet, cadmium exposure is higher than in many other populations, especially outside Asia. Thus, it is still possible that selenium could be of higher importance in populations with toxic exposures (especially pro-oxidants) compared to others, even though this was not evident from the adjusted analyses in the present study.

5.4.3 Epigenetics

The term epigenetics refers to alterations in gene expression that are not due to changes in the genetic sequence, but rather changes in the structure of the DNA. Such changes include DNA-methylation, histone modifications, as well as silencing by microRNAs. Epigenetic mechanisms are thought to be important in mediating the effects of diet on health and development. The most studied mechanism is DNA-methylation, during which a methyl group is covalently added to the 5-carbon of cytosine bases within CpG dinucleotides. This reaction is catalyzed by SAM-dependent DNA-methyltransferases (Anderson et al. 2012). As selenium is methylated prior to excretion, also using SAM as the methyl donor, it may be hypothesized that any competition for methyl groups would result in decreased DNA-methylation with increasing selenium intake. Indeed, a cross-sectional study on human lymphocyte DNA found an inverse association between plasma selenium and DNA-methylation in adults exposed to arsenic-contaminated drinking water in Bangladesh (Pilsner et al. 2011). Experimental studies, on the other hand, have shown that dietary selenium deficiency caused global DNA-hypomethylation of liver and colon DNA (Davis et al. 2000), and that selenium supplementation increases the DNA-methyltransferase activity (Davis and Uthus 2003). Thus, the impact of selenium on DNA-methylation is still unclear and further studies are warranted in this area, including studies on the effect of selenium on other histone modifications and microRNAs.

DNA-methylation is of particular importance for programming during embryonic and fetal development. There appears to be no studies on the importance of selenium on DNA-methylation in early life, but there are human studies that have found links between maternal folate status and other one-carbon nutrients with offspring DNA-methylation. The reported

associations were at specific loci, including imprinted genes, insulin-like growth factor 2, the paternally expressed gene 3 (PEG3), as well as genes related to brain development (Caffrey et al. 2018). To conclude, more studies are needed to clarify any potential role of selenium in epigenetic regulations of gene expression.

5.4.4 Pregnancy outcomes

As selenium has been associated with pregnancy outcomes such as gestational age at birth and birth weight (Mariath et al. 2011), it may be hypothesized that any effect of selenium is mediated through these pregnancy outcomes, since these may also affect brain development (Shenkin et al. 2004). Therefore, we included both birth weight and gestational age at birth in the analyses in **paper I**, and we also tried excluding birth weight to assess whether this resulted in any change in the estimate for selenium (no change observed). In **paper III**, we included birth weight in the 5-year analyses, and gestational age at birth in the 10-years analyses (based on the backwards elimination process for the two models). The positive association between erythrocyte selenium at GW14 and children's cognitive development was present regardless of whether these factors were included in the model, and the estimates did not change by excluding them. Thus, the associations between selenium status in early life and cognitive development seem independent of birth weight and gestational age at birth, implying that the other mechanisms discussed above are more likely to be responsible for the positive associations.

5.5 METHODOLOGICAL CONSIDERATIONS

In all epidemiological studies there are strengths and limitations, and it is important to evaluate whether the findings are precise, valid, and generalizable. Bias can arise at many stages of a study, and are divided in random and systematic error. These types of bias, together with strengths of the studies in the present thesis, are discussed below.

5.5.1 Strengths

The strengths of the studies included in this thesis include the prospective design and the large number of participating mother-child pairs, the repeated comprehensive testing of child development, the many considered covariates, including HOME and maternal cognition, as well as measurement of selenium and other elements using a reliable ICP-MS method. In addition, the use of biomarkers is an advantage as this reflects the internal dose, which might not always be the case when the exposure is estimated from food frequency questionnaires. We also had several biomarkers for selenium (and other elements), assessed at several time points, which enabled evaluation and comparison of the biomarkers of selenium status over time in the present population. The available data on the children's cognitive abilities at different ages strengthens the results, given the consistent findings over time. The ability to adjust for multiple potential confounders was also an advantage, as this shows robustness of the findings (see also section 5.5.3.3). The limitations of the studies included in this thesis are discussed in more detail in the following sections (5.5.2 and 5.5.3).

5.5.2 Random errors

All uncertainties in any type of study are either due to systematic error or random error, where random error is related to the precision of all measurements and is reflected by the width of the confidence intervals. Since this type of error is random from one measurement to the next, it can be minimized by increasing the sample size (i.e. by averaging over a large number of observations). **Paper I** and **III** both included large samples (n=729 and 1408) of mother-child pairs, and thus, the random error is likely to be small in these studies. Indeed, this is indicated by the narrow confidence intervals, particularly in **paper III**. However, when stratifying by child gender, the analyses were indeed more imprecise. Despite stronger estimates among the girls (particularly in **paper III**), this difference was not statistically significant.

For **paper II** and **IV**, the samples were smaller, and any random error might have been more influential in these analyses. Still, the main findings from these papers (e.g. the associations between selenium biomarker kinetics and arsenic and cadmium) appeared precise, and were also present at 4.5 years of age. Thus, the random error was likely small.

As discussed under section 4.4.3, the analytical precision of all analyses of selenium and other elements was high (low CV% and LOD). For hair, the precision was somewhat lower than for the other media (Table 4). This was probably related to the many analytical runs that spanned over almost a year due to the large sample size and complicated procedure of sample preparation. Still, all samples were analyzed in a random order, and the large sample size (n=1330 in the final analyses) appeared to compensate for this variability (**paper III**). Regarding the cognitive outcomes, the inter-observer reliability was assessed both at 1.5, 5 and 10 years, with intra-class correlation coefficients >0.85 at all time points, also indicating low random error.

In addition to sample size, multiple testing also increases the risk of a type of random error. The papers in this thesis include a large number of analyses (multiple exposures and outcomes), which increases the risk of chance findings, i.e. type I error (incorrect rejection of the null hypothesis). However, the outcomes in the present papers (**I** and **III**) are not independent and the hypotheses were pre-defined (except for some predictors in **paper IV**), why this should not constitute a major problem. In addition, the main findings of this thesis (particularly **paper III**) were highly significant, indicating that they probably would have remained so even with false discovery rate adjustment of the p-values. The findings were also consistent over time, which also indicates that the risk of chance findings is low. Finally, incorrectly adjusting p-values for multiple comparisons may in contrast increase the risk of type II error (incorrect acceptance of null hypothesis).

5.5.3 Systematic errors

As opposed to random errors, systematic errors are not related to sample size, but rather depend on the ability to correctly measure exposure and outcome, and the ability adjust for relevant potential confounders.

5.5.3.1 Selection bias

Selection bias may arise when the characteristics of the selected participants are different from those not selected, i.e. when the study sample is not representative of the population it was selected from. In **paper I**, we assessed whether the included participants (n=729 with selenium measurements) differed in any characteristic from those who participated in the follow-up at 1.5 years (developmental assessment) but were excluded from the study due to missing blood samples (n=1383). The participating mothers were older and had somewhat lower BMI and SES, although the differences for all factors were marginal. Other characteristics did not differ. This comparison has now been extended to all women enrolled in MINIMat (n=4436) and to all studies included in the thesis (Table 7).

Table 7. Characteristics [mean ± SD (p-value from Wilcoxon signed-rank test)] of the whole study population and each sample used in the respective papers. N.A., not applicable.

	n in whole sample	Whole study population (N=4436)	Included in paper I (n=729)	Included in paper II (n=207)	Included in paper III (n=1408)	Included in paper IV (n=395)
Mothers						
Age (years)	4422	26.3 ± 6.0	26.7 ± 5.9 (p=0.040)	25.6± 5.9 (p=0.052)	26.6 ± 6.0 (p=0.043)	26.6 ± 6.0 (p=0.40)
BMI (kg/m2)	4435	20.7 ± 14	21.4 ± 23 (p=0.0098)	20.6 ± 3.0 (p=0.19)	20.6 ± 15 (p=0.060)	20.4 ± 2.9 (p=0.13)
SES	4436	3.0 ± 1.4	2.9 ± 1.4 (p=0.0071)	3.3 ± 1.4 (p<0.001)	2.9 ± 1.4 (p<0.001)	3.2 ± 1.3 (p=0.011)
Parity	4436	1.7 ± 5.8	1.5 ± 1.4 (p=0.0062)	1.2 ± 1.3 (p=0.081)	1.5 ± 1.4 (p=0.0011)	1.4 ± 1.3 (p=0.17)
Education (years)	4436	5.0 ± 4.1	4.4 ± 4.0 (p<0.001)	5.7 ± 3.8 (p=0.017)	4.6 ± 4.0 (p<0.001)	5.3 ± 3.9 (0.10)
Children						
Birth weight (g)	3267	2690 ± 410	2690 ± 400 (p=0.48)	2740 ± 370 (p=0.083)	2700 ± 390 (p=0.69)	2750 ± 410 (p=0.0063)
Gestational age at birth (weeks)	3562	38.7 ± 1.8	38.7 ± 1.7 (p=0.72)	38.4 ± 1.6 (p=0.0027)	38.7 ± 1.6 (p=0.17)	38.7 ± 1.6 (p=0.48)
Mental development index at 1.5 years	2112	78.8 ± 12	78.1 ± 13 (p=0.059)	N.A	N.A	N.A
Psychomotor development index at 1.5 years	2112	93.7 ± 13	93.0 ± 14 (0.021)	N.A	N.A	N.A
Comprehension at 1.5 years	2038	38.2 ± 7.7	37.7 ± 7.5 (p=0.030)	N.A	N.A	N.A
Expression at 1.5 years	2037	11.6 ± 6.9	11.2 ± 6.5 (p=0.28)	N.A	N.A	N.A
Verbal score at 5 years	2260	34.2 ± 12	N.A	N.A	32.8 ± 11 (p<0.001)	N.A
Performance score at 5 years	2260	34.7 ± 8.2	N.A	N.A	34.1 ± 7.8 (p<0.001)	N.A
Full score at 5 years	2260	82.4 ± 24	N.A	N.A	79.2 ± 22 (p<0.001)	N.A
Verbal comprehension at 10 years	1530	36.7 ± 11	N.A	N.A	36.7 ± 11 (p=0.95)	N.A
Perceptual reasoning at 10 years	1530	32 ± 12	N.A	N.A	31.8 ± 12 (p=0.35)	N.A
Working memory at 10 years	1530	29.7 ± 6.1	N.A	N.A	29.7 ± 6.2 (p=0.63)	N.A
Processing speed at 10 years	1530	34.1 ± 12	N.A	N.A	34.3 ± 12 (p=0.26)	N.A
Full score at 10 years	1530	132 ± 33	N.A	N.A	133 ± 33 (p=0.41)	N.A

The general characteristics of the included samples were comparable to all women and children included in the trial, although the included women generally had slightly fewer children. The similarities between characteristics of the samples and the whole study population indicate that no selection bias appears to have been introduced when defining the study samples, with regard to these characteristics.

Selection bias may also arise if the exposure or outcomes differ between included and excluded participants. Although the cognitive outcomes could not be compared to the whole cohort (as cognition was not assessed in all children), they could be compared to the larger sample of children with these outcomes assessed (excluded due to missing sample for selenium analyses). At 1.5 years, a total of 2112-2037 children were tested, of which 729 were included in **paper I**. The cognitive outcomes were slightly lower in the study sample, although the differences were very small (<5%). At 5 years, 2260 children were tested, of which 1408 were included in **paper III**. Again, the scores were somewhat lower in the study sample, with very small differences (<5%). Finally, at 10 years, 1530 children were tested and the included children (n=1408) did not differ from the excluded once (p>0.26 for all outcomes).

As the children's selenium concentrations in blood, urine, and hair were analyzed for each respective study it is not possible to compare the selenium status of included children to those in the whole study population. However, the children had a wide range of selenium concentrations in the different media, indicating that both selenium deficient and sufficient children were included in each study. For the maternal selenium status, we have measured erythrocyte selenium at GW14 for a total of 2375 women who are part of various sub-samples of MINIMat (not included in this thesis). Comparing the selenium concentrations between the mothers included in **paper I** (662 women of the 729 included) and **paper III** (n=1408) to the remaining mothers (n=1713 and 967, respectively) showed that the concentrations were higher among women included in **paper I** (158 vs. 152 µg/kg), but lower for those included in **paper III** (152 vs. 157 µg/kg). Given that the difference was in opposite directions for the two studies, and that the average difference was <5%, it seems as though selenium status was not influential for participation. Thus, the risk of selection bias is likely small.

As all pregnant women within the study area were invited to participate, and the participation rate was high, the whole study population of women included in MINIMat (n=4436) is likely representative of the whole study area.

5.5.3.2 Information bias

This type of bias arises when the exposure or outcome classification is incorrect. The use of biomarkers instead of estimated intake from food frequency questionnaires decreases the risk of exposure misclassification, since the internal dose is actually measured instead of estimated. As thoroughly described in section 4.4.3, we did not only have high precision in the selenium measurements, but also high accuracy (Table 4). Still, the variation between

analytical runs introduced some variation also in the selenium concentrations, however, this variation would be expected to be non-differential (random) and would likely drive effects toward the null. Even though the total selenium concentration in plasma or erythrocytes often correlate with the activity of many selenoproteins, analyses of the actual protein levels could have been useful both for confirmation of the selenium status, as well as for evaluation of the mode of action for the beneficial effects.

Regarding the outcomes, they may indeed vary depending on the phycologist performing the assessment. However, as mentioned above, the inter-observer reliability was high, and all analyses were adjusted for tester. In addition, the testers had no knowledge as to the mother's or children's selenium status, and therefore any variation in outcome between testers should not influence the associations.

5.5.3.3 Confounding

When an association is due to factors that are associated with both the exposure and outcome, and not the investigated exposure itself, the reported estimates will become biased. To avoid this potential bias, all analyses of the present thesis were adjusted for a large number of potential confounders.

It could be hypothesized that selenium might be a marker of general nutrition in the present population, and that a better general nutrition is driving the positive association between selenium and cognitive development. However, the associations were apparent also after adjusting for other essential elements (zinc, iodine, manganese) and hemoglobin, as well as BMI (mothers) or HAZ/WAZ (children; markers of chronic and current nutritional status). Still, it could be argued that since selenium is present in fish, the positive associations could be from long-chain fatty acids, also present in fish and known to be beneficial for brain development (Innis 2008). Unfortunately, there was no reliable information regarding dietary intake. To approach this issue, we measured the mercury concentration of children's hair at 10 years, which is known to be a good marker of fish consumption (Castano et al. 2015). Surprisingly, we found no correlation between selenium and mercury in the children's hair (r_S=0.01 p=0.75, n=1330). We also adjusted the analyses in **paper III** for the children's hair mercury concentration at 10 years, and found no changes in the estimates for selenium. Furthermore, for a sub-sample of mothers (n=196) included in **paper III**, mercury had been measured in the erythrocyte fraction from GW14 (Gustin et al. 2017). In this sample, we also found only a weak correlation between erythrocyte selenium and mercury (r_S=0.17, p=0.020). Thus, it appears as if fish is not the main source of selenium intake in the present population, and therefore, any residual confounding from fish consumption is likely low.

As the main source of selenium in infancy is breast milk, and infant formulas have been found to contain low concentrations of selenium (He et al. 2018), it could be argued that the positive association between prenatal selenium status and cognitive abilities would be a proxy for the effects of breast feeding (Horta et al. 2015). We did not have very detailed information regarding breast feeding practice, why this was not included in the papers. However, the

women were asked whether they had breastfed exclusively for 4 months (yes/no), and we have now adjusted the analyses of **paper I** (information available for 482 out of the 729 included mothers) and **III** (information available for 1402 out of the 1408 included mothers) for this variable. There was no difference (<5%) in the estimates for maternal erythrocyte selenium for associations with any outcome at 1.5, 5, or 10 years after this adjustment, supporting that the indicated effect of early-life selenium status was not confounded by breast feeding practice.

Finally, the women enrolled in MINIMat did not smoke or drink alcohol, eliminating any risk of potential confounding from these lifestyle factors.

5.5.4 Generalizability

As shown in Figure 12, low selenium levels are common world-wide, why the findings of this thesis are of interest also for many other populations. We did not find any clear evidence of effect modification by factors such as BMI, SES, or birth weight, which indicates that the results may be applicable also to western populations. Still, the MINIMat study was conducted in rural Bangladesh, where maternal and child undernutrition is still common. The 95^{th} percentile of maternal BMI at GW8 was 25 kg/m^2, indicating that even though there was no apparent effect modification, the variation might not have been large enough to detect such a modification. Indeed, the same theory is applicable to SES and birth weight, and therefore, the results of the present thesis might be generalizable to populations with poor nutrition in particular. In addition, the present population is commonly exposed to pro-oxidants, especially arsenic and cadmium through the rice-based diet and contaminated drinking water. Thus, it is possible that selenium may be extra important in this population due to its antioxidative properties. However, millions of children around the world are heavily exposed to various pro-oxidants, other toxicants, as well as a poor diet.

Some findings may, however, be applicable to most populations. The indicated inverse associations at the highest selenium concentrations are of particular concern for e.g. North America, where the selenium intake is much higher than in e.g. Bangladesh and Europe.

6 CONCLUSIONS

This thesis points to the importance of selenium for cognitive development. In particular, adequate selenium status during pregnancy seems to positively influence children's cognitive abilities even up to 10 years of age. Furthermore, children's own selenium status also seems influential. This is of importance given that millions of people world-wide may be selenium deficient.

The thesis also discusses strengths and limitations of different biomarkers of selenium status and intake, especially among children. New findings include confirmation of the use of selenium in hair (a long-term and non-invasive marker), as well as the shift in biomarker selenium concentrations upon exposure to certain toxicants, and the higher selenium retention in malnourished children.

It should be remembered that high consumption of selenium can have toxic effects. Therefore, intake via food is preferred since it is more sustainable, most often less expensive, and decreases the risk of excessive intake. There were indications of inverse associations between selenium status and cognitive abilities among the present children with the highest selenium concentrations. High intake levels are common in e.g. the U.S. and Canada, and therefore also the inverse associations are of concern for other parts of the world and should be investigated further. In addition, the indicated non-linear relationship in the present thesis, together with findings from similar studies, further supports the suggested lowering of the UL for selenium intake.

Finally, the results may be used by health professionals and authorities in motivating the public (parents in particular) to strive for a varied diet, thereby increasing the chance of reaching an adequate but non-toxic selenium status.

7 FUTURE RESEARCH

The studies and discussion included in this thesis provide evidence for selenium status being important for children's cognitive development, but several questions remain. Future research should focus on the following:

- Confirming the present findings using an experimental design that is focused on selenium, i.e. a randomized clinical trial, preferably in populations with low baseline selenium status, providing different selenium species at low to moderate doses.

- Establishing whether the impact of early-life selenium status on cognitive development is indeed non-linear, and if so, identify the turning point.

- Studying the mechanisms invoved in both the positive and negative effects, e.g. through inclusion of markers for thyroid function, epigenetic changes, and oxidative stress.

- Investigating the role of the many selenoproteins with functions that are still unknown.

- Evaluating to what extent the relationship is dependent on other factors, such as exposure to pro-oxidants or malnutrition.

Regarding interactions with other elements, the assumed complex between selenium and arsenic $(GS_2AsSe)^-$ should be attempted to be confirmed in humans. If present, the toxicological importance and excretory route of such a complex should be assessed.

8 POPULAR SCIENCE SUMMARY

Deficiency of essential minerals is common world-wide and may lead to serious diseases. One of those minerals is selenium, which is found in food items such as meat, fish, and cereals. A daily intake of selenium that is too low is very common in Europe and large parts of Asia. This is due to the low selenium content in the soil, leading to a low content also in plants and animals that we eat.

It has been known for some time that selenium is important for the immune system, fertility, and for the cardiovascular system. However, the interest has lately grown for the importance of selenium for the function of the brain. Among elderly, it has been shown that selenium deficiency is common among people with dementia diseases such as Alzheimer's and Parkinson's, but also among those with milder forms of dementia. The results from these studies suggest that selenium might be important for the cognitive functions of the brain, which are functions involved in thinking, knowing, remembering, and processing of information. However, it is still unknown whether selenium is important for the brain function also early in life, e.g. already during childhood or even fetal stages when the brain starts to develop.

The aim of this thesis was therefore to investigate if selenium is important for proper development of the cognitive functions of the brain, and to study if this is particularly important during certain periods of development. To do this, we measured selenium in biological samples collected from pregnant women in rural Bangladesh. These women participated in a large micronutrient supplementation study, including 4436 women, recruited in 2001-2003. Once their children were born, they were followed-up on several occasions until 10 years of age, and biological samples such as blood, urine, and hair, were collected also from them. During visits to the health care facilities, their cognitive functions were also assessed by testing of their motor skills, language development, memory, and problem solving at both 1.5, 5, and 10 years. The results from such tests are often used to calculate IQ.

We measured the amount of selenium in the biological samples collected from the mothers and children. The amount of selenium in different biological samples reflects the selenium intake over the past days (urine) or months (blood and hair). After statistical analyses, controlled for factors that may affect development, such as stimulation at home, socioeconomic status, growth, and schooling, it turned out that children born to mothers with more selenium in their blood during pregnancy performed better on the cognitive tests both at 1.5, 5, and 10 years of age. Also, children who themselves had higher selenium intake (assessed through the amount in hair) performed better than others at the tests at 10 years, although there was an indication that too much selenium may be dangerous to the brain.

To summarize, this thesis provides new knowledge regarding health effects of selenium early in life. The results indicate that adequate selenium is important for brain development and function, but that the limit for toxic effect may be lower than expected. This knowledge can be used in future revisions of the nutritional guidelines, but should be confirmed by others.

9 POPULÄRVETENSKAPLIG SAMMANFATTNING

Brist på livsviktiga mineraler är vanligt förekommande över hela världen och kan leda till allvarliga sjukdomar. Ett sådant mineral är selen, som finns i bland annat kött, fisk och olika sädesslag. I Europa och stora delar av Asien är det vanligt att befolkningar har ett för lågt intag av selen. Detta beror till stor del på att det finns för lite selen i jorden, vilket gör att växter och djur som vi sedan äter inte innehåller tillräckligt med selen för att fylla vårt dagsbehov.

Sedan tidigare vet man att selen är viktigt för immunförsvaret, fertilitet och för hjärta och kärl, men på senare tid har betydelsen av selen för hjärnans funktion också studerats. Hos äldre har man sett att brist på selen verkar vanligt hos individer med demenssjukdomar som Alzheimers och Parkinson, men också vid mer lindriga demenstillstånd. Resultaten från sådana studier antyder att selen är viktigt även för hjärnans kognitiva funktioner, alltså de processer som involverar tänkande, minne, vetande och hantering av information. Vad man dock inte studerat tidigare är om selen är viktigt för hjärnans funktion och utveckling redan tidigt i livet, såsom under barndomen eller under fosterstadiet när hjärnan börjar utvecklas.

Målet med denna avhandling har därför varit att undersöka om selen är viktigt för utvecklingen av hjärnans kognitiva funktioner, och att studera om det finns vissa perioder tidigt i livet då selen är extra viktigt. För att göra detta analyserades selen i biologiska prover som samlats in från gravida kvinnor i Bangladesh i samband med en större studie som påbörjades 2001 och inkluderade 4436 kvinnor. Efter att deras barn föddes följdes de upp vid flera tillfällen upp till 10 års ålder, och även från barnen samlades biologiska prover såsom blod, urin och hår. När barnen kom till hälsoklinikerna för uppföljning testades också deras kognitiva funktioner såsom rörelseförmåga, språkutveckling, minne och problemlösningsförmåga när de var 1.5, 5, och 10 år gamla. Resultat från sådana tester läggs ofta ihop och används för att beräkna IQ.

I de biologiska proverna som samlats från mammorna och deras barn mättes hur mycket selen som fanns, vilket speglar hur mycket selen de intagit den senaste tiden. Beroende på vilket biologiskt material som används kan man uppskatta selenintaget de senaste dagarna (urin) eller de senaste månaderna (blod och hår). Efter statistiska analyser, kontrollerade för övriga faktorer som kan vara viktiga för utvecklingen såsom stimulering i hemmet, socioekonomisk status, tillväxt och skolgång, visade det sig att barn till mammor som hade mer selen i blodet under graviditeten presterade bättre på de kognitiva testerna både vi 1.5, 5 och 10 års ålder. Även barn som själva hade högre selenintag presterade bättre på de kognitiva testerna, men det fanns också en antydan till att för mycket selen kan vara farligt.

Sammanfattningsvis bidrar denna avhandling med ny kunskap om hälsoeffekter av selen tidigt i livet. Resultaten indikerar att tillräckligt intag av selen verkar viktigt även för hjärnans utveckling och funktion, men att gränsen för när selen blir giftigt kan vara lägre än förväntat. Denna kunskap kan användas vid revidering av näringsrekommendationer för selen, men bör även styrkas ytterligare av andra studier.

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